NOVEL BACTERIAL DIVERSITY IN AN ANCHIALINE BLUE HOLE ON ABACO ISLAND, BAHAMAS

A Thesis

by

BRETT CHRISTOPHER GONZALEZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

Chair of Committee,	Thomas Iliffe
Committee Members,	Robin Brinkmeyer
	Daniel Thornton
Head of Department,	Thomas Lacher, Jr.

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ABSTRACT

Novel Bacterial Diversity in an Anchialine Blue Hole on Abaco Island, Bahamas.

(December 2010)

Brett Christopher Gonzalez, B.S., Texas A&M University at Galveston

Chair of Advisory Committee: Dr. Thomas Iliffe

Anchialine blue holes found in the interior of the Bahama Islands have distinct fresh and salt water layers, with vertical mixing, and dysoxic to anoxic conditions below the halocline. Scientific cave diving exploration and microbiological investigations of Cherokee Road Extension Blue Hole on Abaco Island have provided detailed information about the water chemistry of the vertically stratified water column. Hydrologic parameters measured suggest that circulation of seawater is occurring deep within the platform. Dense microbial assemblages which occurred as mats on the cave walls below the halocline were investigated through construction of 16S rRNA clone libraries, finding representatives across several bacterial lineages including *Chlorobium* and *OP8*. In many blue holes, microbial metabolism of organic matter in the presence of seawater sulfate leads to anoxic and sulfidic conditions at or below halocline. Sunlight penetrating this sulfidic layer allows for *in situ* primary production to be dominated by bacterial anoxygenic phototrophs. Although water column chemistry and molecular genetic diversity of microbial mats in

Cherokee Road Extension Blue Hole were investigated in this study, the full scope of the biogeochemistry of inland blue holes throughout the Bahamas Archipelago is complex and poorly understood. However, these microbial communities are clearly influenced by several factors including solar insolation, terrestrial and marine inputs of oxygen, carbon, and nutrients, water residence times, depth to the halo/chemocline, and cave passage geometry. The biogeochemistry of inland blue holes throughout the Bahamas is so distinctive which makes Abaco Island and the rest of the archipelago valuable as natural experiments, repositories of microbial diversity, and analogs for stratified and sulfidic oceans present early in Earth's history.

To all who have shared special moments in my life;

my friends, family, and loved ones

…this is dedicated to you.

ACKNOWLEDGEMENTS

To everyone who has assisted me throughout my graduate career, I express sincere thanks. Dr. Thomas Iliffe took a chance and accepted me into his lab and personally introduced me to the beautiful anchialine labyrinths which occupy this vast subterranean world. I am forever indebted for all of the amazing adventures over the years throughout the Caribbean. Each expedition was a once in a lifetime opportunity and I appreciate his willingness to consider my participation. Together we have been the first to explore countless caves, finding amazing discoveries. I am here to share these adventures with everyone due to his diligence to ensure safe return from each and every dive. Dr. Robin Brinkmeyer introduced me to the world of microbiology and allowed me to work in her lab. She provided the needed instruments and supplies to make this research possible, yielding remarking findings, and allowing me to carve out a niche of my own in anchialine microbiology. Dr. Dan Thornton enthusiastically served on my committee and mentored me throughout this long journey. His efforts and support in the final steps will always be remembered.

The knowledge I have gained throughout my master's career will always be remembered and is instrumental to whom I am today and what lies ahead in my future.

I am indebted to all of the cave divers who assisted and trained me; you represent the best in the world and it has been a privilege to dive and learn from

you. Gregg Stanton's amazing training provided me the skills and the mental ability and awareness to dive in the most dangerous places on Earth. Tami Thomsen had the remarkable ability to capture the true beauty of these underwater caves. Thank you so much for making your images available to me to use. Brian Kakuk was always willing to lend assistance and instruction to a fellow diver. His involvement provided the much needed field logistical aid throughout all of our Bahamas expeditions.

I would also like to thank the Marine Biology department at Texas A&M University at Galveston which has graciously offered financial support through teaching assistantships throughout my tenure. Special thanks to Drs. Renee Bishop, Jenn Macalady, and John Spear who provided invaluable advice and/or use of their labs for analysis.

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In conclusion I would like to posthumously honor those who in my mind continuously acted as environmental stewards throughout their lives: marine biologist Dr. Mia Tegner and photographer/videographer Wes Skiles. Their work has and will continue to influence so many; they will never be forgotten!

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CHAPTER I

INTRODUCTION: ANCHIALINE BLUE HOLES OF THE BAHAMAS

Introduction

Marine cave ecosystems were once considered to be inhabited only by accidentals (i.e., organisms with no significant cave adaptations that inadvertently enter caves) (Vandel, 1965; Riedl, 1966). However, subterranean environments have recently become recognized for their microbial diversity, generating remarkable discoveries that revealed unique and previously unknown microbial communities (Gibert and Deharveng, 2002; Barton *et al.,* 2004). The research presented here embarks on a new frontier in microbiospeleology, presenting the first look at the bacterial diversity and biological processes within the subterranean aquatic ecosystems (blue holes) of Abaco Island within the Bahamas archipelago. The high degree of vertical stratification within these blue holes gives rise to numerous microhabitats, each with their own potentially unique microbial interactions. Studies of both terrestrial and aquatic caves scattered throughout the world have shown a chemoautotrophic bacterial presence governing the food web (Sarbu *et a.l*, 1996; Pohlman *et al.,* 1997) in contradiction to Dickson's (1975) perception that all aquatic cave ecosystems are detritus based.

This thesis follows the style and format of *The ISME Journal*.

The ability to identify and characterize the microbial diversity within a given cave will not only identify the existence of chemoautotrophy within anchialine ecosystems, but will provide further evidence against the perception that all aquatic cave ecosystems are detritus based.

This opening chapter provides a detailed examination of the unusual characteristics (e.g., geology, hydrology, speleogenesis, and environmental threats) of anchialine blue holes, while research objectives and hypotheses for this study are listed and explained.

Bahamas Archipelago

The Bahamas archipelago (Figure 1.1) with over 700+ islands and cays is located in the Atlantic Ocean southeast of the mainland United States. In addition to a land area of 11,404 km^2, the archipelago has 125,000 km^2 of submerged shallow platform (banks) (Meyerhoff and Hatten, 1974) separated by deep water channels which divides the chain into northwestern and southeastern topographic providences. The northwestern province is comprised of two major platforms, the Little Bahama Bank and Great Bahama Bank, while the southeastern province contains smaller banks (Mullins and Hine, 1989; Carew and Mylroie, 1995a; Sealey, 1995; Roth, 2004). The islands of the northwestern province lie on one of the two major platforms, while the islands in the southeastern province lie on small banks, each separated by deep water (Carew and Mylroie, 1997). Abaco Island (study site) is located on the Little

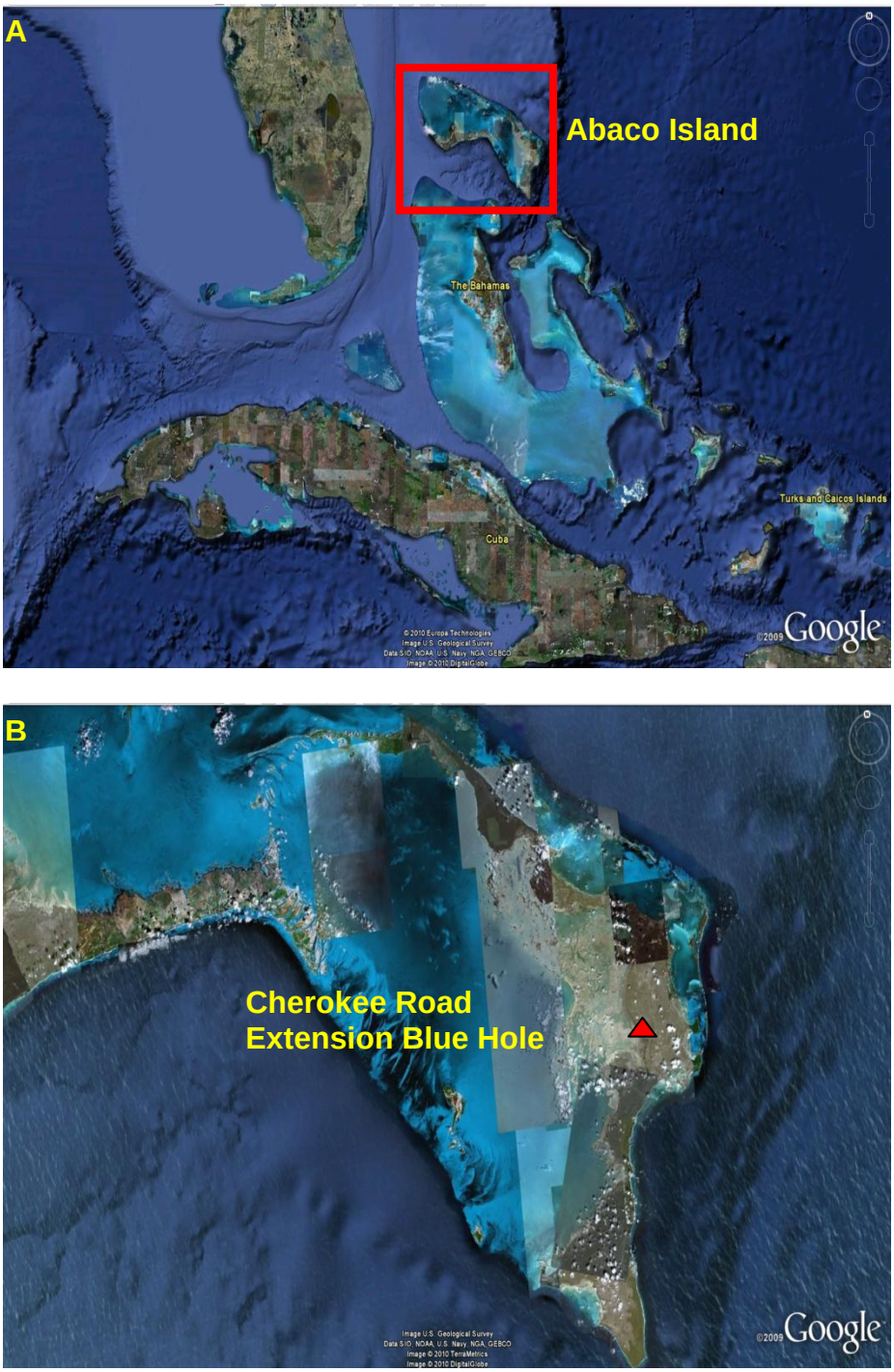


Figure 1.1 Satellite image of the Bahamas Archipelago. A) Relative location to Florida and Cuba as well as bathymetric features such as shallow water platforms and deep oceanic canyons. Red box encompasses study island. B) Abaco Island shown on shallow platform with Cherokee Road Extension Blue Hole marked by ▲ symbol.

Bahama Bank, along with Grand Bahama Island and Moore's Island, and is separated from the other banks by a deep oceanic canyon (Figure 1.2).

Drilling investigations have revealed carbonate oozes, marine shale, and fossilized coral reefs as deep as 5766 meters, suggesting that the Bahamas had a shallow water marine origin (Austin and Schlager, 1988; Carew and Mylroie, 1995a; Sealey, 1995). The platform began forming around 200 Ma during the Mesozoic (Mylroie and Carew, 1990; Sealey, 1995; Buchan, 2000) as a much larger landmass, which included parts of Florida, northern Cuba, the Turks and Caicos Islands, and the Blake Plateau. Sometime in the early Cretaceous (~ 80 Ma), geologic processes triggered large-scale bank-margin retreat triggered by erosion and current scour during lowered sea levels, isolating the Bahamas from Florida and Northern Cuba (Walker, 2006). Additional segmentation may have resulted from seismic activity in the Tertiary based on proximity to the North American/Caribbean plate boundary. This fragmentation also flooded the Blake Plateau, and persistent sinking puts it at its current depth of over 900 meters (Austin and Schlager, 1988; Sealey, 1995). Not long after (late Cretaceous), isolated banks began reemerging the islands of the Bahamas formed.

Four glacial eustatic events occurred during the Quaternary altering the Bahamas bank platforms to their present state (Mylroie and Carew, 1990). Interglacials separated the glacials with periods of climate in the Bahamas very much like present time, with platforms and bank sediments completely submerged (Sealy, 1995). During glacial periods, sea levels dropped upwards

of 120 m exposing the Bahamas platforms and bank sediments. Exposed bank sediments were modified and blown around by atmospheric conditions creating the present day landscape and landforms. The most affected sediments during glacials were oolite (Sealey, 1995). Oolite is the lightest and the most prominent of the fringing sediments, capable of being blown around by winds, forming the first beaches and ridgelands (Sealey, 1995). Surface rocks found throughout the Bahamas Islands date to the Pleistocene and Holocene and have varying marine and aeolian limestone compositions (Mylroie and Carew, 1990; Sealey, 1995).

The Bahamas, like many other carbonate islands, has a characteristic hydrology, controlled by meteoric events and porosity of the surrounding rock. The Bahamian karst is distinctive and varies significantly from that of continental Florida (Vacher and Mylroie, 2002) in that each carbonate unit has undergone exposure to the harsh environment of the submerged mixing zone. Beneath the island, freshwater meets and mixes with submerged marine water. This forms a freshwater lens that is thickest in the interior of the island (Raeisi and Mylroie, 1995). This mixing of water masses has aggressive dissolutional properties and aids in the forming of karstic features such as flank margin caves.

Mylroie *et al.,* (2001) proposed and developed the 'Carbonate Island Karst Model (CIKM)' to geologically explain these solutional processes on small carbonate islands such as the Bahamas, Bermuda, Guam, Jamaica, Puerto Rico, and the Mariana Islands. There are four geomorphic categories of the

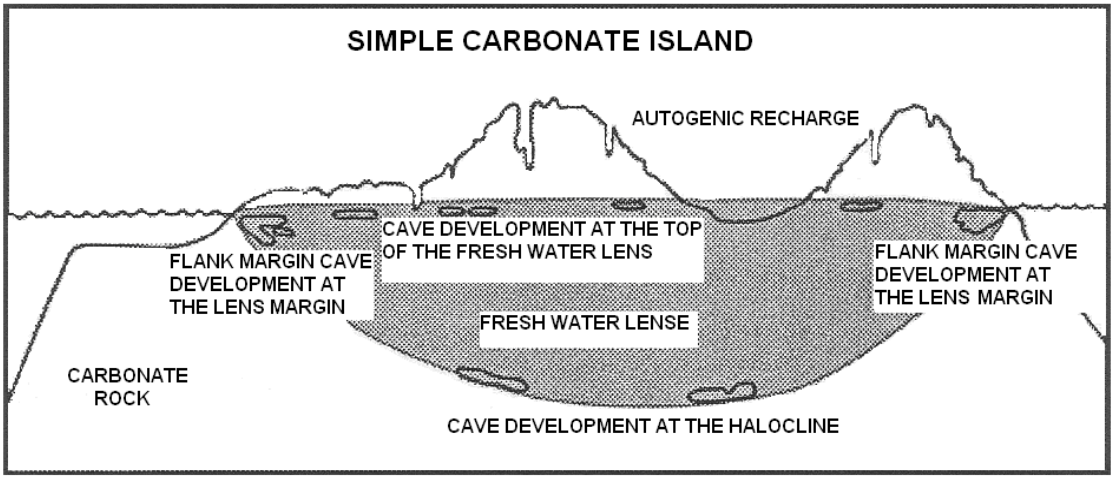


Figure 1.2 Simple carbonate island model (Mylroie *et al.,* 2001).

CIKM determined by position of the carbonate basement, sea level, and island surface (Mylroie *et al.,* 2001; Roth, 2004). Islands in the Bahamas are classified as 'simple carbonate islands' (based on the CIKM) in which the geology and hydrology is dependant solely on carbonate rocks penetrating below the freshwater lens (Figure 1.2).

Bahamian karst is believed to be eogenetic (young carbonate rocks without deep burial) under continuous diagenesis. Dissolutional sculpturing has formed karren throughout the island's limestone, along with a variety of other karstic features including caves, cracks, depressions, and blue holes (Carew and Mylroie, 1995a; Sealey, 1995). Karren can be described as dissolutional sculpturing due to meteoric water (Roth, 2004). It is best described as etchings on the underlying rock layers which grades down into the epikarst below. The etchings range in scale from millimeters to meters and are separated by sharp

ridges. The karren observed throughout the Bahamas is distinct from other settings due to constant contact with sea spray (Mylroie and Carew, 1995).

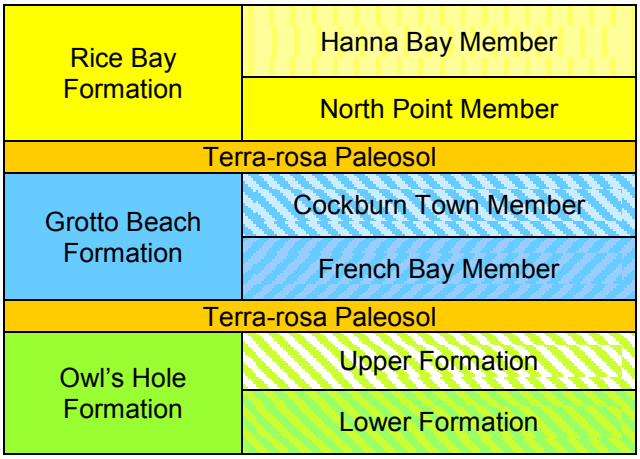


Figure 1.3 Physical stratigraphy of the Bahamas.

The stratigraphy of the Bahamas (Figure 1.3) is based on rocks from numerous islands, with San Salvador Island as the prime reference area (Carew and Mylroie, 1995b). Recent studies (Walker, 2006) have indicated that the stratigraphy identified from San Salvador is applicable to Abaco Island. Surface rocks throughout the archipelago date from the Middle to Late Quaternary (1.8 Ma – 10 ka) and are divided into smaller subunit depositional packages (Roth, 2004). The Pleistocene Owl's Hole Formation is the oldest surface rock, consisting of eolianites covered by terra-rosa paleosol and then overlain by either a second terra-rosa paleosol or subtidal deposits (Carew and Mylroie, 1995b). Terra-rosa paleosols are red colored fossil soils, buried within sedimentary or volcanic deposits. These terra-rosa paleosols are derived from

the Sahara desert, carried into the Caribbean by trade winds and deposited by precipitation during glacial eustacy (Herwitz *et al.,* 1996). The reoccurrence of terra-rosa paleosols within the stratigraphy of the Bahamas indicates extended exposure to sea level high and low stands. Exposed Owl's Hole rocks are extensively micritized (microboring and carbonate precipitation within boreholes associated with edolithic activity causing grain welding to occur) at the surface with portions weakly cemented below. As limestone weathers, impurities such as clay become deposited and iron oxide forms within, giving the rock its reddish color. Further paleomagnetism investigations within the Owl's Hole Formations showed distinct division into upper and lower formations (Roth, 2004).

Overlying the Upper Owl's Hole Formation is another Pleistocene derived formation known as the Grotto Beach Formation, which is in turn subdivided into French Bay and Cockburn Town members. The Grotto Beach Formation is the most widespread depositional package throughout the islands (Carew and Mylroie, 1995b). The French Bay member is a transgressive-phase eolian deposit formed as sea levels rose, increasing shelf areas (Roth, 2004), while the Cockburn Town member includes regressive phase beach and eolian deposits, easily identified by herring-bone cross bedding and asymmetrical ripples (Carew and Mylroie, 1995b). Often abundant marine fossils such as corals and mollusks are embedded in this member. A terra-rosa paleosol is occasionally present, yet often times eroded away.

Recent layering during the Holocene (10 ka) is known as the Rice Bay Formation, which is subdivided into North Point and Hannah Bay members. Rice Bay formations consist of eolianites and beach facies deposited during the current sea level high stand (Carew and Mylroie, 1995b). North Point members are primarily peloidal (i.e., prolific non-skeletal carbonate grains) with meniscus calcite (i.e., precipitations at or near the grain contacts in the vadose zone) and occasional marine cements (Carew and Mylroie, 1995b). The Hannah Bay member is composed of peloidal/bioclastic (i.e., fragmented organic remains) grainstones associated with low-Mg calcite cements. Rocks such as these were deposited with current sea levels (Carew and Mylroie, 1985; 1995b) and can be found throughout the archipelago.

Abaco Island

The Abaco Islands of the northern Bahamas are situated on the Little Bahama Bank and consist of two main islands, Great and Little Abaco, as well as numerous smaller cays and islands. Abaco Island has an area of 1690 km^2 and a population just over 14000 (Sealey, 1995). It is heavily karstified and exhibits numerous karstic features including karren, blue holes, anchialine pools and ponds, pit caves, banana holes, and flank margin caves (Walker *et al.,* 2008). Blue holes associated with this island are deep (100+ m) and contain extensive horizontal passages, which gives insight towards the geologic development and glacial eustatic events on the island.

Anchialine Environment

Anchialine habitats are known to exist throughout the world often underground, forming a halocline where freshwater mixes with the intruding seawater. Predominantly these habitats lie within tropical limestone islands, but can also be found in volcanic rock and along continental margins. Anchialine habitats can occur as small water filled pools and depressions (Hawaiian Islands), coastal tectonic faults and fissures (Galapagos Islands), flooded lava tubes (Canary Islands), solutionally dissolved limestone conduits (Yucatán, Mexico), and deep vertical shafts (Bahamas) (Stock *et al.,* 1986; Iliffe, 1992; 2000; Kornicker and Iliffe 2000; Kornicker *et al.,* 2002).

Anchialine ecosystems are similar to those of deep abyssal plains, sharing absolute darkness, isolation, paucity of food, stable temperatures, and low dissolved oxygen (Martens and Danielopol, 1999; Aden, 2005; Hobbs, 2005; Hüppop, 2005). This extreme and complex ecosystem represents one of the last unexplored frontiers in terms of biodiversity, ecology, and evolutionary processes. Biologists have just begun to scratch the surface of our understanding of subterranean ecosystems.

Within the last two decades, cave diving biologists have uncovered hundreds of novel invertebrate species, never seen before, with remarkable adaptations to lightless, food poor environments nearing anoxia (e.g., Kornicker and Iliffe 2000; Kornicker *et al.,* 2002; Iliffe and Bishop, 2007). The potential for continued discovery appears limitless.

Invertebrates and fish inhabiting these isolated and extreme environments are considered “living fossils,” showing little evolutionary modifications from their ancestors (Iliffe, 1992). The diversity of stygobitic animals (entirety of the lifecycle restricted to subterranean groundwater) seen today arose due to niche partitioning, which occurred prior to the closure of the Tethys Sea (Boxshall and Jaume, 1999). Stygobitic organisms are restricted to subterranean groundwater habitats and characterized by loss or reduction of eyes and pigment, often accompanied by attenuation of the body and appendages. Previous investigations have concluded that stygobitic species share ancestry with taxa now inhabiting the deep-sea (Jaume and Boxshall 1995; Martens and Danielopol, 1999).

Holthius (1973) coined the term “anchialine” from the Greek word ‘*αγχί-άλός* (= near the sea) to describe these unique bodies of water, which have “…no surface connection to the sea…” These systems are controlled tidally by subterranean passages and contain both salt and brackish waters. As cave diving investigations have amplified in the past decade, our understanding of anchialine habitats have expanded and the concepts behind the term ‘anchialine’ has thus been revised. The International Symposium on the Biology of Marine Caves (1984) suggested modifications of the definition to its present verbiage:

> *Anchialine habitats consist of bodies of haline waters, usually with a restricted exposure to open air, always with more or less extensive subterranean connections to the sea, and showing noticeable marine as well as terrestrial influences.*

Blue holes

Blue holes are some of the largest karstic features found throughout the Bahamas. These water-filled sinkholes are subdivided into oceanic and inland blue holes (Bottrell *et al.,* 1991; Kornicker *et al.,* 2002), both formed by dissolution of limestone during periods of ocean eustasy.

Oceanic blue holes usually have horizontal cave passage extending under the sea floor as deep, vertical cracks running parallel to the platform edge (Kornicker and Iliffe 2000; Kornicker *et al.,* 2002). These ocean holes have strong reversing tidal currents, and act as hydrological conduits transporting water across islands between ocean and bank sides (Iliffe, 2000). Imbalances often occur due to lag between bank tides and those of the open ocean tides (Iliffe, 1992; Iliffe and Bishop, 2007), as a result, oceanic blue holes “suck” and “blow” water depending on the phase of the tidal cycle.

Inland blue holes are deep circular shafts that widen and bell out into subterranean abysses (Kornicker and Iliffe 2000; Kornicker *et al.,* 2002). Many of the inland blue holes have little to no horizontal passages partially due to original collapse or subsequent detrital fill.

Inland blue holes (Figure 1.4) of the Bahamas contain tidally influenced layers of brackish and marine waters, separated by a halocline. These meromictic (layers of water which do not intermix) water bodies are characterized by the presence of several strong density interfaces each associated with complex physico-chemical profiles (Stock *et al.,* 1986; Bishop *et al.,* 2004; Seymour *et al.,* 2007). Rock permeability influences the mixing and overall thickness of the halocline (Iliffe, 2000). Blue holes which exist within the island's interior exhibit a dampening of tidal oscillations, which in turn decrease mixing, thus, tapering the halocline (Iliffe, 2000; Beddows *et al.,* 2001). Primary cave development exists at this boundary where mixing corrosion cause limestone dissolution to occur (Back *et al.,* 1986). Occasionally, hydrogen sulfide (H_2S) may form at the halocline when exogenous input of particulate organic matter is high and naturally occurring bacteria deplete the already low levels of dissolved oxygen.

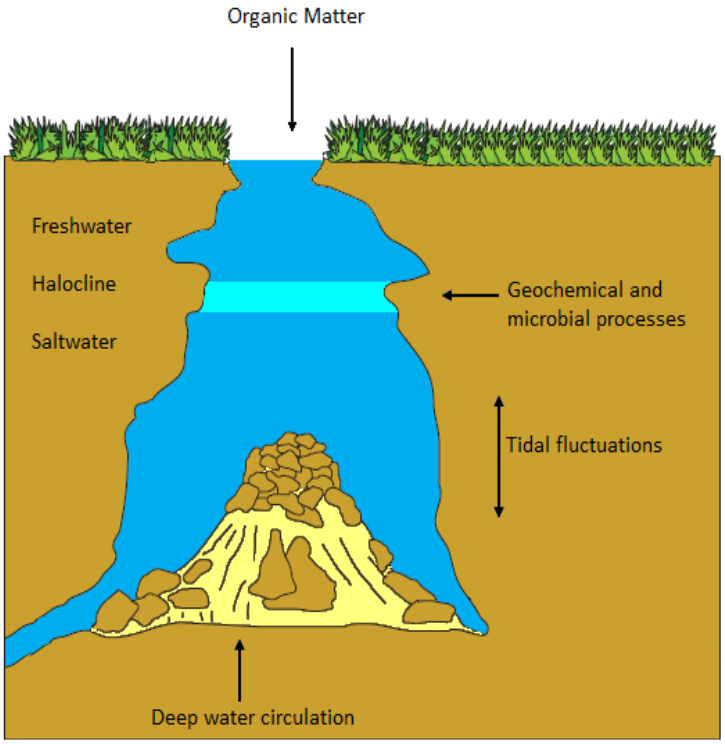


Figure 1.4 Typical blue hole showing water stratification as well as entrance and breakdown mound. Courtesy of Curt Bowen and Advanced Diver Magazine expedition reports.

Ghyben-Herzberg lens

The Gyben-Herzberg principle states where fresh groundwater floats on underlying seawater, there is 40 m (or ft) of freshwater below sea level for every meter (or foot) above sea level (Vacher, 1997). As meteoric water percolates through the pore spaces, the weight of the water displaces the underlying seawater forming a profile resembling a lens. This principle basis its assumptions that both the freshwater and seawater are hydrostatic immiscible fluids (Vacher, 1997). Freshwater has a density of 1.0 and is lighter than the

density of saltwater, 1.025, creating a ration between the two of 41:40. The creation of this principle is essential in determining the availability of freshwater on an island. The mathematical formula to determine availability of freshwater is the following where h_s is the depth of freshwater below sea level, h_f is the depth of freshwater above sea level and .025 is the difference between the two densities:

$$h_s = h_f / .025$$

Realistically the principle is problematic since neither of the water layers are static, nor is there a sharp boundary between the two layers. The transition zone marks the separation of the two layers, forming brackish waters (halocline). The Gyben-Herzberg principle must not be overlooked when discussing the hydrogeology of carbonate islands, yet its use must be applied with care. Vacher (1997) suggests a rewording of the Gyben-Herzberg principle to read: *it's a relationship that attempts to find the depth of the sharp freshwater-saltwater interface that would be present if there were no mixing*.

Water chemistry

The water column in anchialine blue holes is highly complex, exhibiting distinct stratification within the physico-chemical parameters. These same water parameters can be seen in water-filled caves in Yucatán, Mexico, Bermuda, Western Australia, and other Caribbean Islands. With each parameter uniquely

demarcated, microhabitats can be identified in intervals as small as 20 cm (Seymour *et al.,* 2007).

Salinity. Water found in anchialine blue holes is an extension of the surrounding subsurface groundwater, much like other caves and springs. A freshwater lens is formed when freshwater overlays denser saltwater forming distinct boundaries. The upper boundary of the lens has little salt content and originates from high precipitation. Freshwater layers of blue holes further inland are thicker than blue holes near the coast due to greater inland recharge (rainfall) and reduced tidal disturbances. Below the halocline, the water is of fully marine salinity.

Temperature. Salinity stratified anchialine blue holes exhibit little temperature variance amongst seasons in each of their distinct water masses. Temperature maximum and minimum are found either at surface or at depth depending on geographic location and water circulation. Temperature increases are usually associated with the halocline and a steadying or leveling off of temperature is normally observed with depth (Iliffe, 2000). Deeper waters within blue holes owe their increased temperatures to either surface evaporative cooling or heat plumes from deep geothermal activity (Iliffe *et al.,* 1983).

Acidity. In Bahamian blue holes, well defined pH and oxygen minimums occur at the halocline where microbial respiration decreases oxygen levels and produces CO_2 (Iliffe, 2000). Since haloclines are known areas of increased limestone dissolution (Pohlman *et al.,* 1997; Roth, 2004), the corrosive water within the mixing zone may explain abrupt pH shifts. Blue holes that contain H_2S below or at the halocline may have abrupt pH shifts reaching values below 6.0 due to sulfur oxidizing bacteria in contrast to blue holes lacking H_2S.

Dissolved oxygen. Surface waters have nearly saturated dissolved oxygen (DO) levels due to constant atmospheric exchange and perturbation (Iliffe, 2000). DO levels at the halocline show marked decreases, with continued decline as depth increases. DO levels at depth often are <1 mg/L and rarely exceed dysoxic concentrations. Enhanced microbial oxidation at the pycnocline may drive anchialine hypoxia (Iliffe, 2000).

Oxidation-reduction potential. Surface and near surface waters above the halocline are in continuous contact with atmospheric oxygen; therefore are in an oxidizing state, showing positive ORP values (Hasenmueller *et al.,* 2006). ORP is a measure of the tendency of a chemical species to acquire electrons and becoming reduced. Water below the halocline is constantly mixing with reducing sediments along the cave floor, causing continued reduction to be

present. The increase in reducing tendency may be contributed by hypoxic conditions from H_2S or high microbial densities (Hasenmueller *et al.,* 2006).

Circulation within anchialine systems

Seawater circulation within carbonate platforms is controlled by the driving mechanisms which draw water in; the margin topography, and the permeability (Martin and Moore, 2008). Seawater circulation within these platforms is in response to a hydraulic head formed due to differences in fluid density and sea surface elevations (Whitaker and Smart, 1993). Anchialine systems occur in basaltic lava and limestone, both of which have a subsurface connection to the sea that responds to tidal fluctuation and circulation. Emphasis will be placed on circulation of anchialine systems found within limestone strata.

Whitaker and Smart (1993) identify four models illustrating the mechanisms driving circulation of saline ground waters in carbonate platforms; 1: Elevation Potential, 2: Buoyant Circulation, 3: Reflux, and 4: Thermal Convection.

Differences between open ocean and shallow carbonate banks can cause tidal pumping to occur where large quantities of water are being exchanged throughout the margins of rimmed carbonate platforms. Other forms of elevation potential can be due to diurnal tides, wind, waves, or oceanic currents. Long lasting circulation from an elevation potential is heavily dependent on platform

morphology and irregularities such as rimmed margins and tidal flat levees (Whitaker and Smart, 1993).

Buoyant circulation will occur on an emergent platform when meteoric recharge leads to the formation of a stratified water column with a distinct freshwater lens present overlying seawater (Kohout, 1960; Whitaker and Smart, 1993). Areas of meteoric recharge will have a similar hydrological environment (freshwater atop saltwater) to that of the inland blue holes in the Bahamas and cenotes of Yucatán. Dispersive mixing and discharge of hydrodynamically unstable brackish water along the platform margins is in response to barometric pressure, meteoric recharge, and tidal oscillations (Kohout, 1960; Whitaker and Smart, 1993). Platform morphology and climatic regime heavily control buoyant circulation; thus the northern Bahamian Islands such as Abaco would be expected to be more active due to a better developed freshwater lens (Whitaker and Smart, 1993).

Density driven downward movement (reflux) of saline water is caused by evaporation increasing surface salinity (Whitaker and Smart, 1993). Downward displacement of less dense water will continue until stability is reached, equal to oceanic basins, at which point lateral flow can occur (Whitaker and Smart, 1993). The mechanics of this system was originally designed to explain isolated environments such as hypersaline lakes and other restricted bodies of water where seawater circulation is restricted. Hypersalinity in enclosed water bodies is usually due to aridity and high rates of evaporation. Due to meteoric variability

found in the Bahamas, hypersalinity can present itself. Simms (1984) using models demonstrated that only a small scale increases in salinity is needed to induce large-scale reflux of waters throughout carbonate platforms.

Convective circulation of oceanic waters through carbonate platforms was discovered in the sub-surface waters of South Florida by Francis Kohout, which yielded the concept of “Kohout Convection” (Kohout, 1960; Simms, 1984; Whitaker and Smart, 1993; Martin and Moore, 2008). Kohout convection occurs in carbonate platforms where a horizontal density gradient forms between cold oceanic water and warmer porewater, heated by geothermal gradients (Simms, 1984). As cold water is drawn into the interior of the platform, a cold water wedge is formed upward displacing warmer interior waters. Geothermal heat warms this cold water wedge causing a reduction of the overall density thus allowing upward convective flow (Simms, 1984; Whitaker and Smart, 1993; Martin and Moore; 2008). Upward movement is constrained by the freshwater lens causing lateral reemergence of warmed saline groundwater will be through submarine springs and fractures along the platform and shelf edges (Simms, 1984; Martin and Moore, 2008).

The scale and steepness of the platform and associated margins is important in determining what mechanism can exist to drive circulation. Circulation tends to favor larger platforms where a well developed freshwater lens can form and temperature gradients exist between inland bodies of water and open ocean (Whitaker and Smart, 1993). Increased circulation is often

associated around platforms with rimmed shelves and steep margins due to increased horizontal temperature gradients. The porous nature of rimmed margins provides inlets and channels for saltwater to flow through. Permeability of these margins is associated with past positions of the fresh water lens or halocline where active dissolution once occurred and which now may act as conduits for saline groundwater flow (Whitaker and Smart, 1993; Martin and Moore, 2008). The same can be true of fractures found around steep rimmed margins.

Investigations of groundwater in carbonate platforms by Whitaker and Smart (1993) and Martin and Moore (2008) envisaged a single driving mechanism controlling water circulation. Contradictory evidence suggests that complex balances of two or more processes, which may even act to reinforce each other are needed for active circulation of saline groundwater in carbonate platforms (Whitaker and Smart, 1993).

Organic Matter Sources

Primary cave food webs are thought to be autochthonous, with little outside organic input, where chemoautotrophic bacteria constitute the base level of the short stygobitic food chain (Pohlman *et al.,* 1997; Koch, 2001). Movile Cave, a geothermal cave in Romania, is entirely supported by sulfide-oxidizing bacteria, as are submarine caves in Italy where heated sulfurous springs provide nutrient input. In both mentioned caves, primary production is chemoautotrophic

(Sarbu *et al.,* 1996). Bahamian blue holes are rarely associated with readily apparent geothermal sources; therefore biogeochemical processes must arise by other means. Inorganic carbon sources occur in anchialine blue holes primarily from the cave itself ($CaCO_3$ walls) and from biogenic production of CO_2 (Pohlman *et al.,* 1997). Isotopic data obtained by Pohlman *et al.,* (1997) from Yucatán caves shows that chemoautotrophic bacteria may provide enough endogenous material to support two to two and a half distinguishable trophic levels within anchialine caves. Similar studies by Southward *et al.,* (1996) looking at isotope ratios of organisms in submarine caves showed that bacterial contributions in the diet ranged between species anywhere from 0-100%. Because cave food webs are so compressed, survival within such trophic poverty involves evolution of specialized genes and metabolic pathways (Sket, 1996; Koch, 2001).

Consequently, exogenous sources of carbon may be present from blue hole entrances and overlying soil (Pohlman *et al.,* 2000). Research done in Yucatán cenotes (Pohlman *et al.,* 1997) showed that dissolved and particulate organic carbon from overlying soils and detritus percolates in with rainwater, contributing to the overall carbon sources.

Extremophiles

Extremophiles are organisms which thrive in an extreme environment whereby survival is difficult and resultant adaptations are necessary in order to

persist (Rothschild and Mancinelli, 2001). These harsh conditions may be of a physical extreme (such as temperature, radiation, or pressure) or a geochemical extreme (such as desiccation, salinity, pH, DO, or ORP) (Rothschild and Mancinelli, 2001). It has been argued that organisms which live in biological extremes (such as nutritional extremes, population density, parasites, or prey) should also be considered extremophiles. Lack of light and a paucity of dissolved oxygen and organic nutrients, combined with various geochemical parameters (pH, ORP, H_2S) make anchialine caves an extreme environment. Animals that have established a niche under such conditions are referred to as extremophiles. Anchialine stygobites (both micro and macro) have a distinct suite of adaptations to the extreme cave environment and are by definition extremophiles.

Environmental Threats

The freshwater contained in Bahamian blue holes throughout the archipelago is of primary importance for the surrounding ecosystems both above and below ground. Several native species of flora and fauna depend heavily on the availability of these holes to provide safe, reliable water. With the push for development of tourism throughout the archipelago, subterranean ecosystems are at an increased risk of contamination associated with urbanization and development. Freshwater is a vital commodity, exploited throughout the islands through wells, boreholes, and trenches, and is utilized for drinking and irrigation.

Sudden booms in the tourism industry combined with the lack of effective environmental laws and practice, has adversely impacted blue holes and groundwater bodies throughout the archipelago.

Solid waste disposal throughout the Bahamas is an environmentally difficult problem to solve. The common practice of “out of sight, out of mind” has caused irreversible contamination of inland blue holes (Iliffe and Bishop, 2007). The island’s high water table (1-3 m below the surface) limits excavation for landfills, thus above ground solutions are commonplace. Nearly all landfills operate without liners or wastewater catchments. Percolating water or leachate penetrates the porous limestone contaminating the groundwater below (Beddows *et al.,* 2001). The Harrold Road Landfill in New Providence, Bahamas has operated for over 25 years atop a water table a mere 0.5-2 m below (http://www.iadb.org/index.cfm?lang=en). Studies done by the Solid Waste Management Program of the Bahamas have revealed leachate plumes are absent in the shallow freshwater, but are heavily concentrated in the deeper saltwater (Commonwealth of the Bahamas 1998).

In addition to above ground contamination with solids and runoff, liquid waste is rarely treated and often times pumped directly underground. Domestic waste is dealt with by on-site disposal or communal sewage soakaways (septic tanks) combined with a disposal well or drainage field (Buchan, 2000). Less developed areas often have improperly working septic systems not conformed to

building standards (Cant, 1996). Lesser-developed islands of the Bahamas still use latrines or discharge directly into nearby lagoons or bays.

Deep well injection of liquid wastes is a common practice in the Bahamas. Injections occur through boreholes into the saltwater zone between 58-189 m (Sealey, 1995). Injected waste disposal can introduce a variety of pathogens as well as nitrates, detergents, heavy metals, and pharmaceuticals, all which can result in reduced dissolved oxygen and the formation of toxic H_2S (Iliffe and Bishop, 2007). All methods of waste disposal within the Bahamas are inadequate, and evidence of groundwater contamination is mounting in urban areas (Buchan, 2000).

Further development of the Bahamas tourist industry means increased construction of resorts, golf courses, and harbors. Construction of resorts and other large buildings threatens the stability of underwater caves due to the building's support pilings. Golf courses require large quantities of water for irrigation and heavy use of nitrogen rich fertilizers that eventually leach into underground caves and water sources causing eutrophication. Construction of harbors involves dredging waterways, which threatens underlying cave passages. It is of the utmost importance that exploration and research continue on blue hole systems to aid in the development of conservation policy and to further our knowledge and understanding of the biological world we live in before it is lost by anthropogenic encroachment.

Thesis Research

A recent expedition to investigate known and unknown anchialine blue holes on Abaco Island, Bahamas has shed light on the diversity of cave microbes and the various forms in which they exist (Gonzalez pers. obs., 2006). As observed by divers, a several centimeter thick, orange gelatinous bacterial mat was present on the cave walls below the halocline in Cherokee Road Extension Blue Hole. Bacterial mats are rarely seen or not present in other nearby blue holes. To date, bacterial mat communities of this sort are only known from a few islands. It is not known exactly how, and why this selection occurs. Samples of the mats were collected to assess the microbial diversity, and water quality parameters were measured to obtain a more detailed insight of abiotic factors governing the presence of these mats.

Previous investigations of the microbiology of anchialine caves (e.g., Schwabe and Herbert, 2004) have been limited to microscopic characterizations based on cell morphology and enrichment cultures, with a lack of molecular data. As sequencing capabilities improve, microbiologists now have a way to define organisms through molecular phylogeny (Pace, 1997). It is unknown whether bacteria follow this same trend as invertebrate evolution in caves, evolving into discrete species based on geographic isolation and resource availability.

Inference of phylogenetic similarities to previously studied chemoautotrophic bacteria will provide important information about food webs

and overall geomicrobial ecology. With the ability to understand and identify base level constituents in anchialine ecosystems, conservation efforts, as well our understanding of cave ecology and biodiversity, will be heightened. Results from this investigation have broadened our perception of microbial diversity within the concept of increasing divergences, and the potential for discovery of new and heretofore unknown lineages.

Objectives

The overall goal of this research was to use molecular methods to identify and quantify diversity in bacterial mats located at depth below the halocline within Cherokee Road Extension Blue Hole, Abaco Island, Bahamas. The primary objectives of this thesis were as follows:

i. Develop a working protocol for microbial DNA extractions from mat and sediment samples obtained from anchialine blue holes. This protocol will facilitate bacterial investigations from subterranean habitats around the world.

ii. Create 16S rRNA clone libraries for phylogenetic determination of the bacterial community occurring within mats from Cherokee Road Extension Blue Hole.

iii. Extrapolate functional ecology of the mat based on its bacterial phylogenetic groups to determine how anchialine microbial mats affect overall ecological processes.

iv. Based on the bacterial groups determined by 16S rRNA clone libraries, deduce if blue hole ecosystems revolve around detrital influences or that of chemoautotrophy.

Statement of hypotheses

The data collected was used to evaluate the following null hypotheses regarding microbial diversity in anchialine habitats:

- H_0: Anchialine caves have monospecific bacterial assemblages
- H_0: Bacteria found in caves consist of previously identified taxa that are commonly associated with terrestrial soil habitats
- H_0: Bacteria found in caves are cave specific

CHAPTER II

HYDROLOGICAL PARAMETERS GOVERNING CHEROKEE ROAD EXTENSION BLUE HOLE

Introduction

Caves, partially or fully flooded with sea water, occur in karstic terrain found along tropical coastlines throughout the world (Sket 1996, Iliffe 2000). These unusual and understudied environments can extend for several meters or for several kilometers and are distinct from other marine habitats by having no photosynthetic oxygen production outside of the entrance pool, restrictive vertical mixing, and a continuous paucity of dissolved oxygen and nutrients. Termed 'anchialine', these systems characteristically contain vertically stratified fresh and salt water under tidal influence (Humphreys, 1999; Pohlman *et al*., 2000; Seymour *et al*., 2007). Density based vertical stratification results from salinity gradients and the absence of wind- or wave-inducing mixing (Iliffe, 2000). Individual variability of water chemistry and passage configuration can been seen between sites, islands, and continents. Inhabitants of these caves are usually dominated by crustaceans that are specially adapted to live in such extreme environments. Endemism is common amongst the stygobites seen in anchialine caves; often times these species are restricted to a single cave. Regardless of the differences, all anchialine systems connect to the ocean

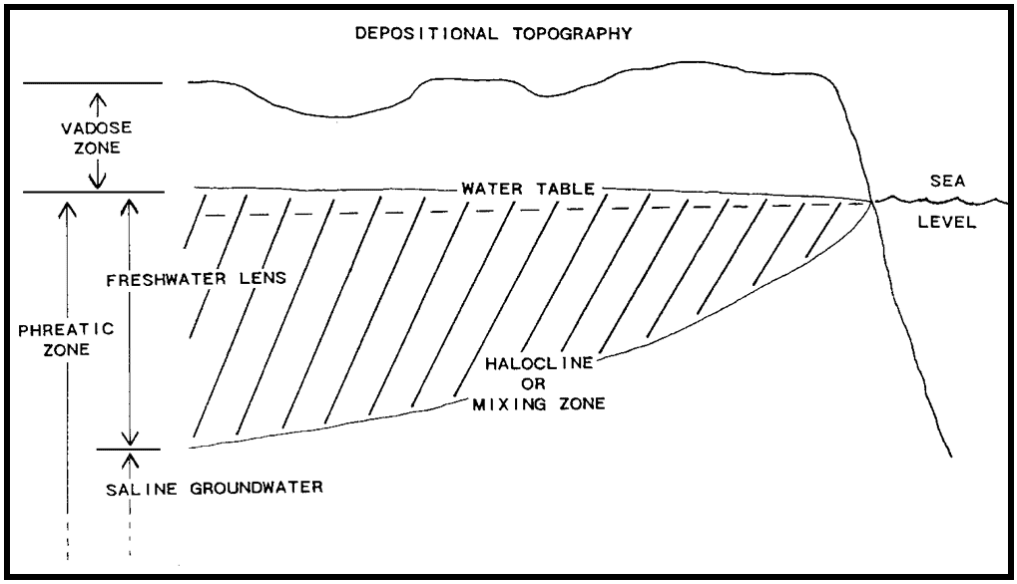


Figure 2.1 Diagrammatic representation of a freshwater lens (after Mylroie and Carew, 1995). Distinct water masses including the halocline or mixing zone commonly associated with carbonate islands and platforms are identified.

through subterranean voids and interstitial spaces (Stock *et al*., 1986; Seymour *et al*, 2007).

Carbonate islands are underlain by density-contrasted groundwater bodies of water, with the surface of the groundwater referred to as the water table (Figure 2.1) (Roth, 2004). Due to the density differences and restricted mixing, less dense freshwater floats atop underlying marine water creating a lens-shape. In the Yucatán Peninsula of Mexico where surface streams are absent and annual rainfall is high, the freshwater lens is extensive, ranging in thickness from 10 m near the coast to over 60 m in the interior. Playa del Carmen on the Caribbean coast of Yucatán has an average rainfall of 1500 mm yr^{-1} (Wilson, 1980). Abaco on the Little Bahama Bank in the northwestern Bahamas receives ~1500 mm yr^{-1} of rainfall annually (Mather, 1988), similar to

that of parts of the Yucatán. Like Yucatán, the depth of the freshwater lens throughout the Bahama Islands is not static. Freshwater lens depth on carbonate islands is controlled by rock permeability, meteoric recharge, distance from the coast, and possibly oceanic tides (Iliffe, 2000; Roth, 2004). Hydrologically, an ideal island would have the greatest thickness of freshwater in the center of the island, tapering towards the edges in the form of a lens (Raeisi and Mylroie, 1995; Roth, 2004). Freshwater lens development in islands across the Bahamas archipelago is different due to dissimilar topographic and geographic settings. Islands in the southeastern Bahamas tend to be arid with freshwater found only in small pockets under dune ridges, while in the northwestern, the islands receive more rainfall and the freshwater lens extends under the entire island (Carew and Mylroie, 1997).

The blue holes found throughout the Bahamas archipelago are similar to anchialine systems found elsewhere around the world. Seymour *et al*., (2007) found that anchialine sinkholes in Australia exhibit the same characteristic meromictic water columns as are found in Bahamian Blue Holes and Yucatán Cenotes. Regardless of the geographic setting, freshwater rests atop the intruding saltwater, creating a mixing layer or halocline between the two distinct water masses. Depth of the halocline is a function of the annual rainfall and the overall distance from the coast (Whitaker and Smart, 1997). Evidence supporting this relationship is documented throughout the Bahamas and Yucatán. Inland Blue holes from Grand Bahama and Abaco Island on the Little

Bahama Bank show a deepening of the halocline as distance from the coast increases. Similar results of haloclines deepening with increased distance from the coastline can be found throughout the cenotes of Yucatán.

Anchialine blue hole entrance pools have characteristics similar to limnetic systems (Seymour *et al*., 2007). Both are well lit (within entrance pool), and capable of sustaining phytoplankton and zooplankton. However, the water in karstic anchialine blue hole pools continuously exchanges with the subterranean seawater, a pattern dissimilar to that observed under normal limnetic settings (Seymour *et al*., 2007). The porosity and permeability of the surrounding limestone regulates the intrusion of groundwater. Continuous mixing of mildly acidic freshwater within the bedrock allows dissolution to expand the pore spaces. As water percolates through the soil, CO_2 is absorbed into the water transforming it into a weakly acidic solution of carbonic acid (H_2CO_3). Blue holes of the Bahamas typically are found in older limestone where solutional channels, crevices, and caverns have enlarged allowing nearly infinite permeability of the underlying water (Iliffe, 2000).

Given the nature and connection of anchialine systems to the sea, one might expect to find physico-chemical profiles similar to those of the surrounding ocean. However, the profiles associated with Cherokee Road Extension Blue Hole, as well as those of neighboring blue holes, are highly complex, and bear little resemblance to ocean parameters. While inland blue holes on Abaco as of now lack humanly explorable connections to the ocean, they still respond to tidal

oscillations. Variations in physico-chemical parameters between inland blue holes and the ocean are dependent on flow paths of both seawater and meteoric freshwater through modern carbonate platforms (Simms, 1984; Martin and Moore, 2008). Mineral ratios (e.g., Sr^{2+}/Cl^{-}, Ca^{2+}/Cl^{-}, and Mg^{2+}/Cl^{-}) in saline groundwater samples from boreholes on Grand Bahama Bank indicate modern seawater can flow at depths nearing 200 m below the sediment-water interface (Swart *et al*., 2001).

Meteoric recharge involves water gain through precipitation minus water loss through evapotranspiration. Recharge zones are controlled by permeability of the limestone surface and island topography (Martin and Moore, 2008). In contrast to freshwater inputs, seawater has an unlimited source from the surrounding ocean waters, yet flow paths are dependent on underlying processes drawing water into the porous and permeable Pleistocene carbonate platform (Sims, 1984; Whitaker and Smart, 1993; Stoessell *et al*., 2002; Fleury *et al*., 2007; Martin and Moore, 2008). Coastal aquifer models by Ghyben (1889) and Herzberg (1901) assume that salt water in coastal aquifers is static. However, Cooper (1959) argues that salt water is not static and is in a perpetual cycle flowing to and from the ocean through cracks in the limestone into the zone of diffusion at the halocline. Brackish water is hydrodynamically unstable and tends to move towards island margins where it can be discharged through diffusion zones (Kohout, 1960; Whitaker and Smart, 1993).

Discharge from diffusion zones occurs at coastal springs and seeps in karstic areas (Cooper, 1959; Gonzalez pers. obs., 2007). Compensatory flow of seawater into the system from depth is required to replace saltwater lost through mixing zone discharge (Cooper, 1959; Whitaker and Smart, 1993). Dives performed with the Johnson Sea-Link on the flanks of San Salvador Island support this deep water circulation model by locating cave openings at depths greater than 100 m below sea level along the platform margins (Martin and Moore, 2008). Additional supporting evidence of deep-water circulation is found in blue holes reaching depths greater than eustatic low stands. Deans Blue Hole on Long Island, Bahamas is 202 m deep (Wilson, 1994). It has been suggested that Deans Blue Hole consists of a cavern formed atop other deeply buried ancient caverns, developing one continuous cave as island subsidence occurs. Other possible explanations for the depth of Dean's Blue Hole involves the cave forming atop a major fault where corrosive gasses (H_2S) caused solutional expansion to occur to its present depths. Regardless, Bahamian blue holes and other karstic dissolution features that formed during glacial eustacy are ideal conduits to circulate waters found in present day blue holes (Whitaker and Smart, 1993; Stoessell *et al*., 2002).

Kohout Convection may explain the circulation within anchialine systems, whereby denser cold seawater migrating inward through the porous limestone margin from the surrounding ocean depths, acts like a wedge, displacing warmer groundwater heated by geothermal flux (Simms, 1984; Whitaker and Smart,

1990; Reed *et al*., 2005). The horizontal density gradient of deep cold seawater and that of warmer water from the interior of carbonate platforms is enough to create an open convection cell, uplifting seawater within the interior of the platform where it will discharge from submarine springs along the present day shelf and margins (Simms, 1984; Whitaker and Smart, 1990; Stoessell *et al*., 2002). Using a conservative mixing model, Whitaker and Smart (1990) proposed that the water discharging from an oceanic blue hole offshore from North Andros Island, Bahamas must have originated from depths of 260 m in either the Tongue of the Ocean or the Straits of Florida. This presents unequivocal evidence supporting Kohout Convection of deep ocean water through a carbonate island platform. Such deep circulation may explain the unusual physico-chemical parameters found in Cherokee Road Extension Blue Hole, Abaco Island, Bahamas.

Methods

In March 2006, a two-week scientific cave diving expedition was conducted to survey the cave fauna of Abaco Island as part of a National Science Foundation grant from the Biodiversity Surveys and Inventories Program (DEB-0315903).

One of the caves investigated during this trip was Cherokee Road Extension Blue Hole, an inland blue hole located in a pine forest 20 km southwest from Marsh Harbour on Abaco Island. This cave is a collapsed

sinkhole reaching depths of 104 m but has little horizontal linear passageway. Like all anchialine blue holes, Cherokee Road Extension is influenced by the semi-diurnal tides. Since this Blue Hole is 2.2 km from the bank side (west) and 6.5 km to the ocean side (east) of Abaco Island, it behaves similar to neighboring Sawmill Sink Blue Hole, lagging behind the coastal tides by approximately an hour (Steadman *et al*., 2007). The nearly circular surface pool at Cherokee Road Extension Blue Hole has a diameter of 11 m and a circumference of 30.4 m (Figure 2.2a). Dissolutional undercutting and collapse has created a bell-shaped profile (Figure 2.2b). Collapse of the carbonate rock from the former cave ceiling has formed a talus cone with its peak at a depth of about 50 m. Cherokee Road Extension Blue Hole has well defined freshwater and saltwater zone separated by a halocline initiating at 13.7 m. A hydrogen sulfide layer has also formed at the halocline (diver observed cloudy layer with H_2S smell). Well-delineated limestone strata occur within the bedrock walls, often with secondary, subaerially formed carbonate speleothems. Speleothem formations are present on ledges below the halocline, while massive algal stalactites which bend toward entrance light are present at shallower depths.

Water quality parameters were measured at Cherokee Road Extension Blue Hole using an YSI 600XLM Multi-Parameter Water Quality Monitor Sonde calibrated to manufacturers specifications (Appendix 1) before deployment. Resolution and accuracy of the unit's probes is presented in Appendix 1. Using

Figure 2.2a Brian Kakuk (Bahamas Underground) standing beside the entrance pool of Cherokee Road Extension Blue Hole, Abaco, Bahamas. Photo courtesy of Tamara Thomsen, Diversions Scuba, WI.

Figure 2.2b Looking up from depth in Cherokee Road Extension Blue Hole, Abaco, Bahamas. Photo courtesy of Tamara Thomsen, Diversions Scuba, WI.

a two second logging interval, depth, temperature, conductivity, dissolved oxygen (DO), pH, and oxidation reduction potential (ORP) were measured while the sonde was slowly lowered till contact with substrate. The sonde was held horizontal slightly below the surface of the water to equilibrate the sensors before lowering. To avoid measuring an artificially mixed water column, divers entered the water only after the measurements were performed. Further diving investigations in the system has explored this blue hole 104 m, greater than measured during the deployment of the sonde.

Data gathered from all measured variables was downloaded from the sonde and exported to Microsoft Excel. Measured variables were graphed as a function of depth. Conservative mixing diagrams were created using constituent concentrations versus salinity

Results

The physico-chemical profiles of the water column in Cherokee Road Extension Blue Hole are characteristic of anchialine waters, with pronounced stratification in all parameters similar to neighboring inland blue holes.

Temperature and salinity

Cherokee Road Extension Blue Hole has a marked thermocline beginning at approximately 12.5 m where the temperatures increased from about 21.4 to 24.4°C at 54.9 m (Figure 2.3). Temperatures continue to slightly increase with

depth below this layer. The entrance pool (Figure 2.1) is in direct sunlight throughout the day and under optimal conditions; sunlight penetrates well below the halocline. Note the slight increase in temperature at the surface warmed by insolation.

Salinity in Cherokee Road Extension Blue Hole is relatively constant at 0.77 ppt from the surface until a distinct halocline is reached at 13.7 m (Figure 2.4). Beneath the halocline, the salinity increases to approximately 31.56 ppt at 25.6 m, and then continues gradually increasing with depth to approximately 33.87 ppt at 54.9 m, nearly approaching the average salinity of the local seawater (35.5 ppt).

Dissolved oxygen, pH, and ORP

Dissolved oxygen levels in the entrance pool of Cherokee Road Extension Blue Hole were significantly higher compared to those levels at depth (Figure 2.5). DO concentration dropped from 5.28 mg/L (surface) to 4.63 mg/L at 16.5 m, then dropped rapidly below this depth. At a depth of 32.28 m, DO levels fell below 1 mg/L, and decreased further to 0.39 mg/L at 54.9 m.

The pH gradually declined from 7.54 at the surface to 6.8 at 16.5 m (Figure 2.6). Within the H_2S layer (~25.2 m), the pH reached a minimum value of 6.26. Below this depth, the pH gradually increased to 6.5 at 54.9 m.

ORP levels were distinctly bimodal, ranging from +70.6 to -142.2 (Figure 2.7). Surface waters showed higher ORP values (oxidation), while at deeper

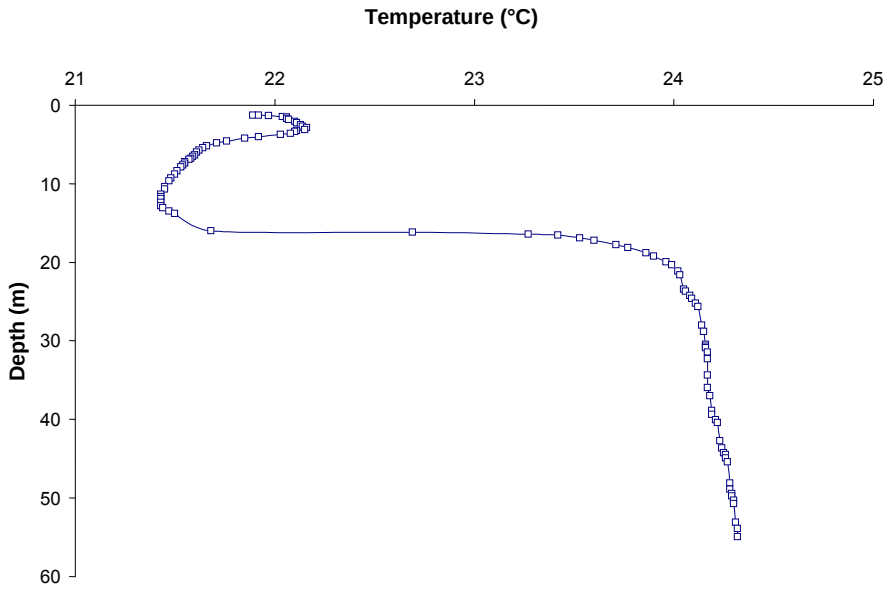


Figure 2.3 Vertical profile of temperature in Cherokee Road Extension Blue Hole. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and 55 m water depth.

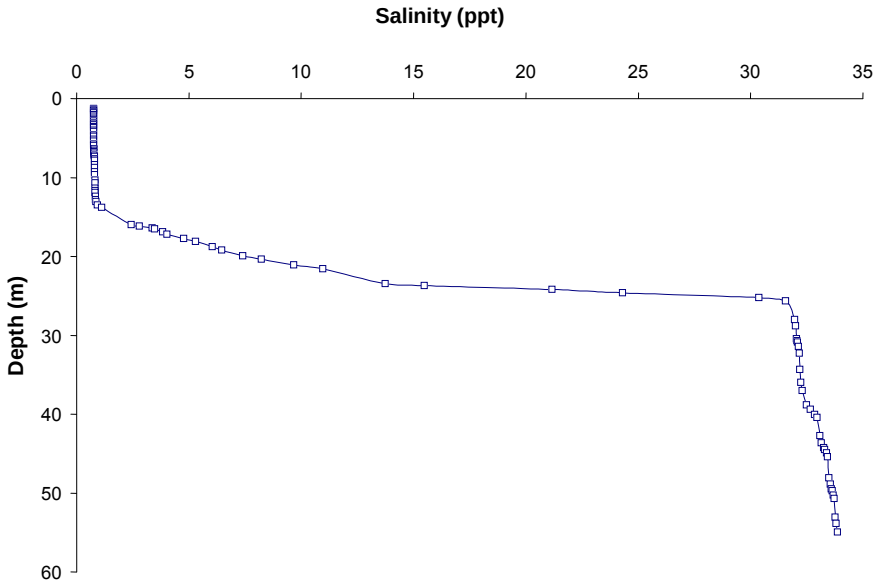


Figure 2.4 Vertical profile of salinity in Cherokee Road Extension Blue Hole. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and 55 m water depth.

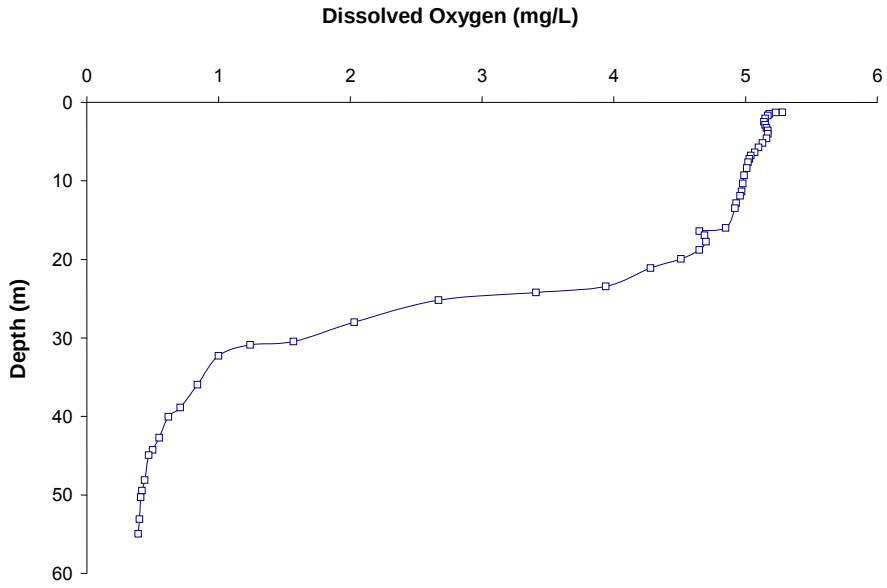


Figure 2.5 Vertical profile of dissolved oxygen in Cherokee Road Extension Blue Hole. Individual measurements, indicated by □ symbol, were taken at 4 second intervals between the surface and 55 m water depth.

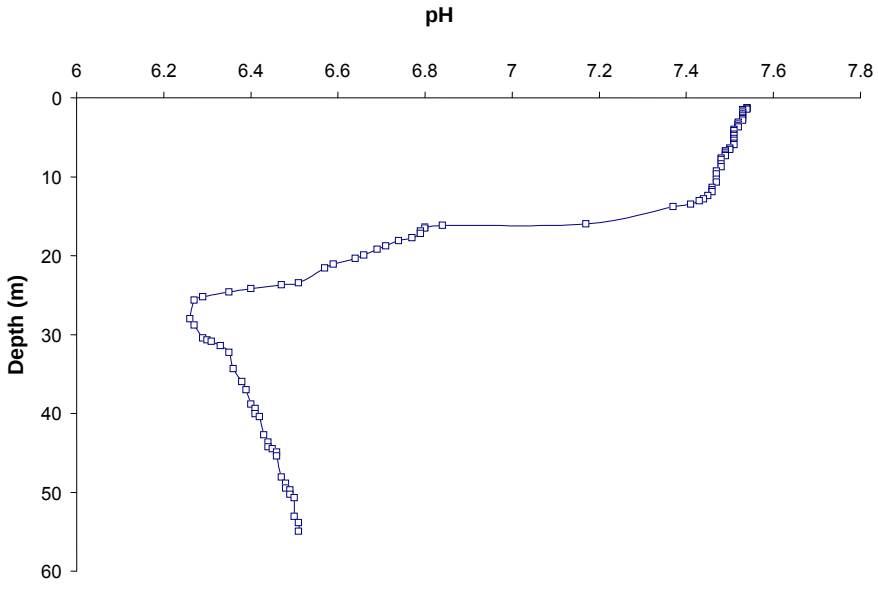


Figure 2.6 Vertical profile of pH in Cherokee Road Extension Blue Hole. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and 55 m water depth.

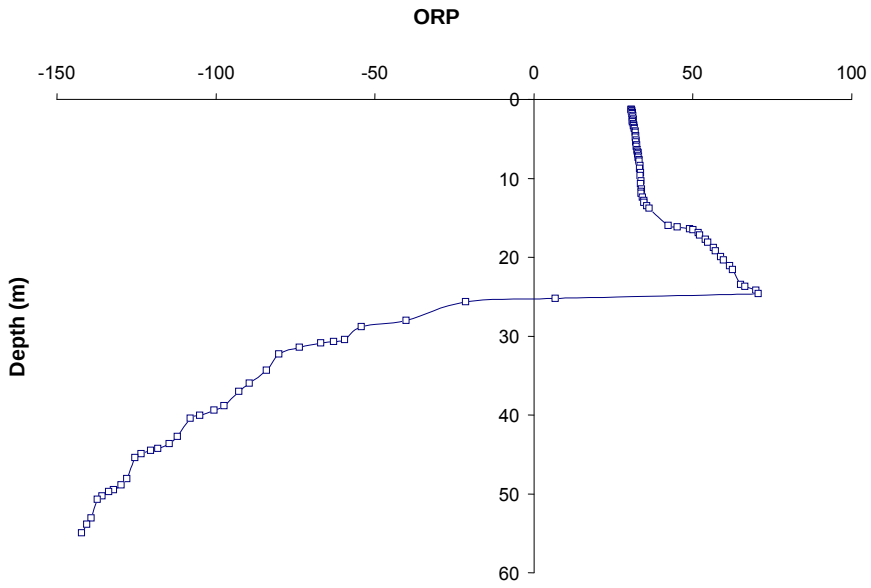


Figure 2.7 Vertical profile of ORP in Cherokee Road Extension Blue Hole. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and 55 m water depth.

depths, negative values (reducing conditions) were observed. The highest ORP occurred at 24.5 m, while below this depth, values dropped dramatically.

Throughout the complex water column in Cherokee Road Extension Blue Hole, multiple stratification layers occur for each hydrologic parameter. Plots of the constituent concentration versus salinity (mixing diagrams) can further elucidate the ongoing processes within the water column at Cherokee Road Extension Blue Hole. Dashed lines represent conservative mixing (Figures 2.8a – 2.10a), convex or concave deviation from the dashed lines indicate whether

the system is acting as a net source or a sink for the given constituent (Officer, 1978; Officer and Lynch, 1981; Kemp, 1989). Mixing diagrams were constructed in the same fashion as those Humphreys (1999) plots from Bundara Sinkhole, Australia.

Water present in Cherokee Road Extension Blue Hole has an overall warming trend since entering the groundwater as indicated by non-conservative mixing between salinity and temperature (Figure 2.8a). Upon investigation of the individual water masses (Figure 2.8b-d) a slight cooling affect is present in the freshwater and saltwater layers. Non-conservative mixing is also observed within the halocline which appears to be the source of hydrogen ion production (Figure 2.9c). DO concentrations also show non conservative mixing overall (Figure 2.10a), however across the halocline (Figure 2.10c) the oxygen loss appears to be conservative, quickly changing to non conservative in the saltwater layer.

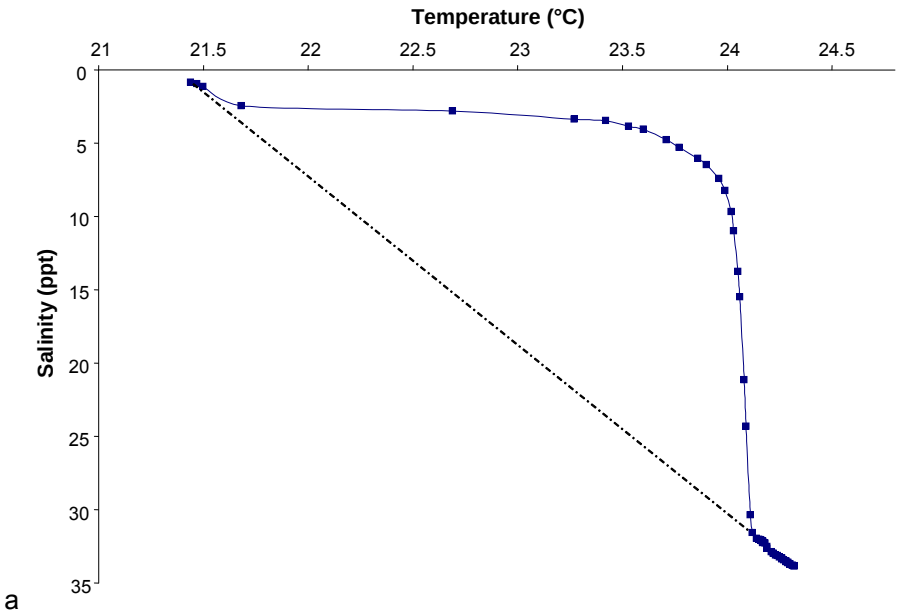
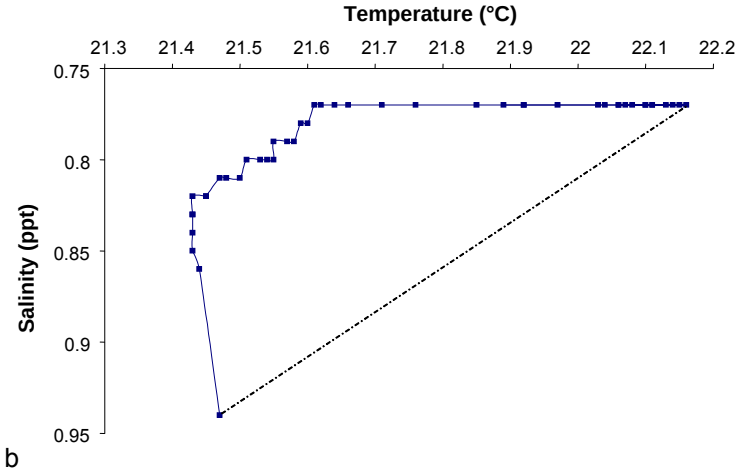


Figure 2.8 Salinity plots of temperature from Cherokee Road Extension Blue Hole. Dashed lines between salinity and end members represent conservative mixing hypothesis. a) Mixing diagram through all three water masses. Plots (b-d) show mixing diagrams from each of the different water masses present: b) freshwater layer, c) mixing zone or halocline, d) saltwater layer. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and maximum water depth.



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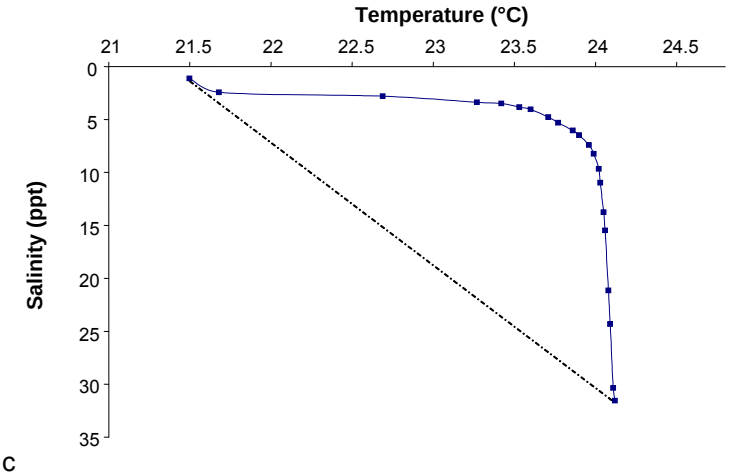

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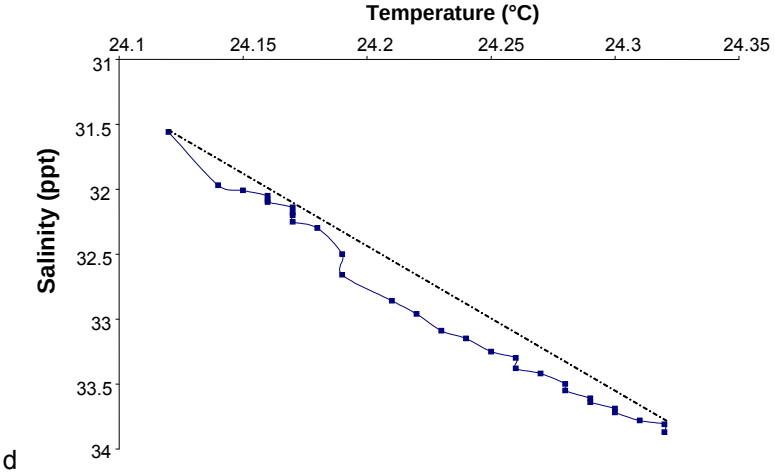

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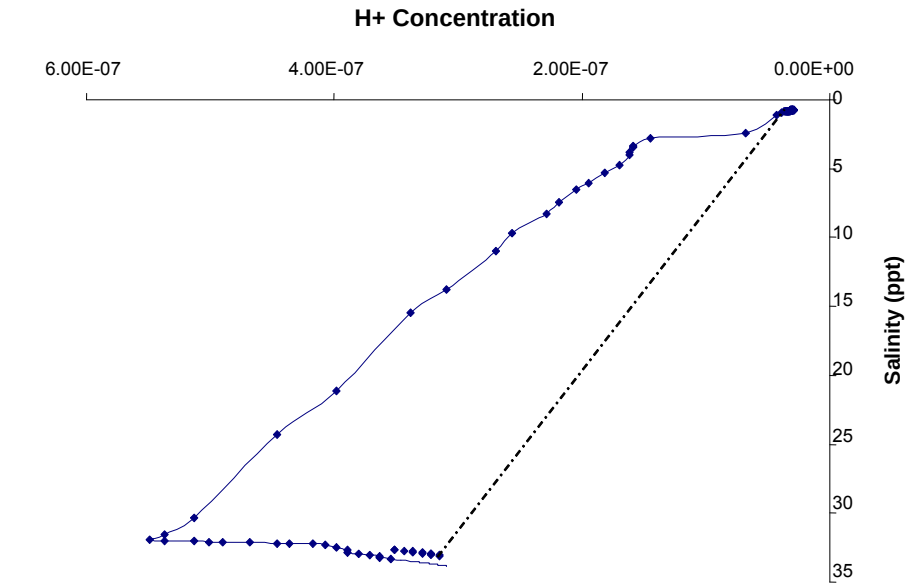
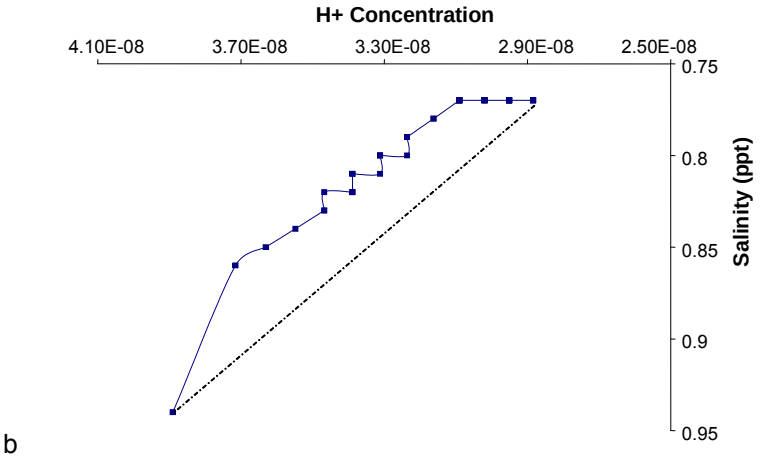


a

Figure 2.9 Salinity plots of hydrogen ion concentrations from Cherokee Road Extension Blue Hole. Dashed lines between salinity and end members represent conservative mixing hypothesis. a) Mixing diagram through all three water masses. Plots (b-d) show mixing diagrams from each of the different water masses present: b) freshwater layer, c) mixing zone or halocline, d) saltwater layer. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and maximum water depth.



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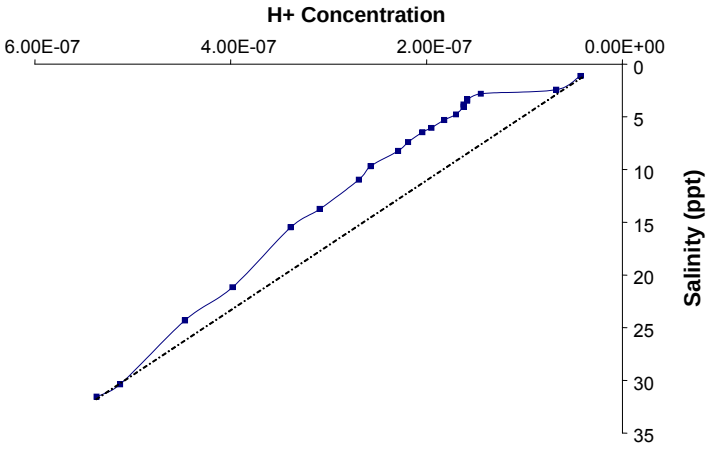

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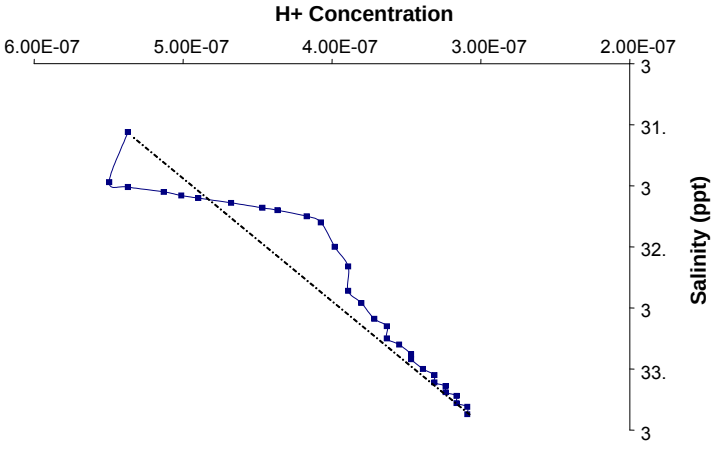

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Figure 2.9 Continued.

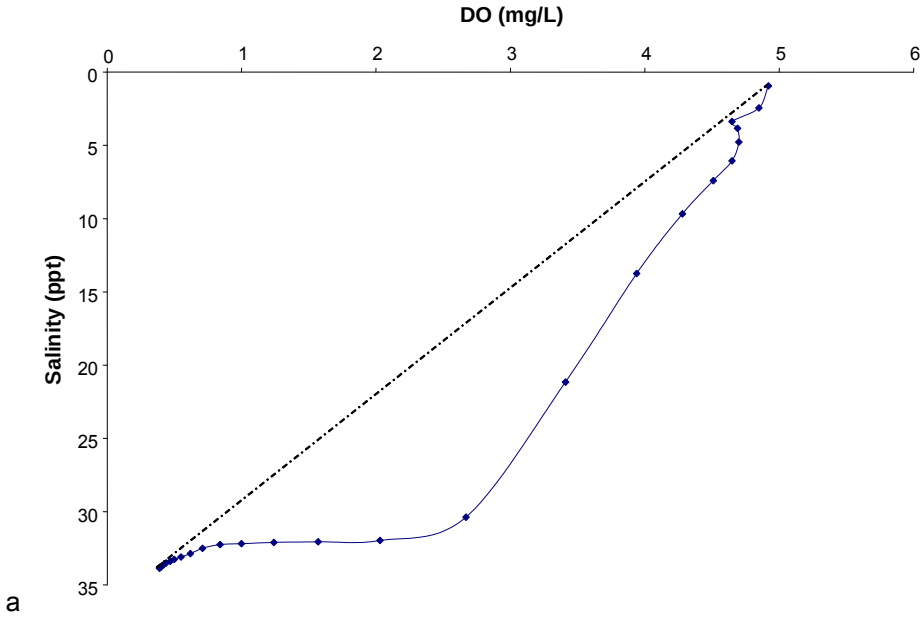
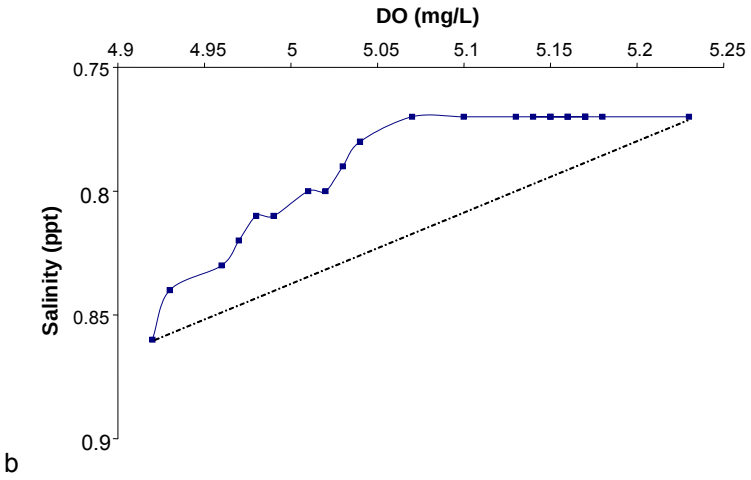


Figure 2.10 Salinity plots of dissolved oxygen from Cherokee Road Extension Blue Hole. Dashed lines between salinity and end members represent conservative mixing hypothesis. a) Mixing diagram through all three water masses. Plots (b-d) show mixing diagrams from each of the different water masses present: b) freshwater layer, c) mixing zone or halocline, d) saltwater layer. Individual measurements, indicated by □ symbol, were taken at 4 second intervals between the surface and maximum water depth.



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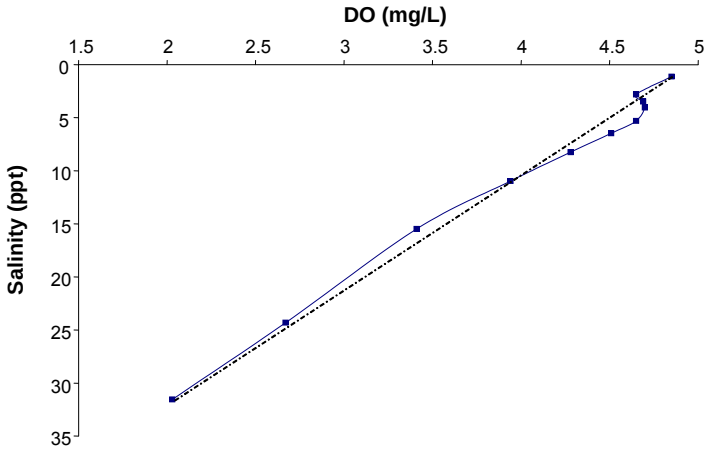

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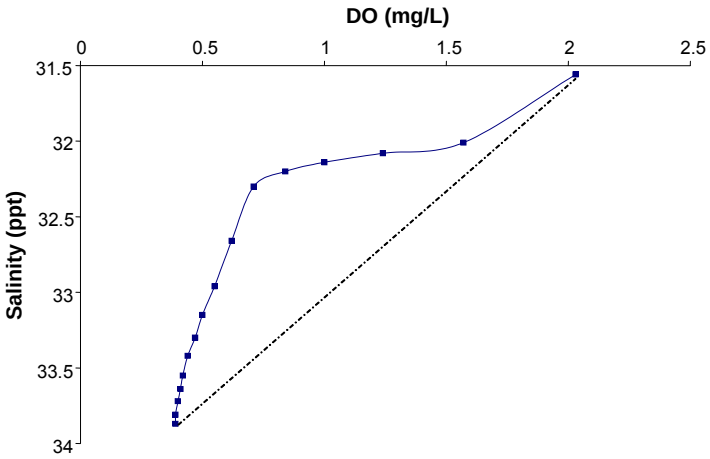

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Figure 2.10 Continued.

Discussion

Subterranean estuaries

The activity of biological communities within estuaries and similar habitats is a function of the physical and chemical processes that provide and transform raw material into energy within and among individuals (Kemp, 1989). Estuaries, by definition, are sites where coastal bodies of water have free connection to the sea and are measurably diluted with freshwater from land drainage (Pritchard, 1967). Although anchialine caves (blue holes and cenotes alike) have not been normally categorized as estuaries, they are clearly sites where underground meteoric freshwater meets intruding seawater. Moore (1999) introduces the concept of a “subterranean estuary”, defined as the mixing zone between groundwater and seawater in a coastal aquifer. Based on numerous field observations, I argue that anchialine caves can be considered subterranean estuaries since they display some of the most important features of coastal estuaries (Moore pers. com., 2009).

There are two possible outcomes when fresh river water mixes with seawater in coastal estuaries. Either dissolved or particular constituents behave conservatively (concentrations changed only by dilution in a linear relationship) or marked transformations occur due to physical, chemical and/or biotic processes (forming concave or convex lines when graphed) (Kemp, 1989). Similar results to those seen in coastal estuaries should occur during transition from the freshwater layer, through the halocline, into the saltwater layer in

anchialine caves. It is important to note that since there are only a few studies on anchialine caves, and even fewer where mixing diagrams are used (Pohlman *et al*., 1997; and Humphreys, 1999) using estuarine mixing diagrams may have some bias. Traditionally mixing diagrams for traditional estuaries are made along the estuary, e.g., from a series of surface samples where possible to identify the two end members, freshwater at one end seawater at the other end, with a gradient of mixing between the two members (Thornton pers. comm., 2009). The true hydrology of karstic terrain is unknown since there are multiple sources of bother freshwater and seawater, however, taking this into account, using estuarine mixing diagrams is the best way to look at biogeochemical processes occurring throughout the system.

Vertical stratification

Anchialine caves in Australia, Bermuda, Yucatán, and the Bahamas among others have vertical stratification and water quality parameters comparable to that of Cherokee Road Extension Blue Hole. Similarities between inland sites within each of these countries and those of Abaco include a freshwater or brackish layer above a well defined halocline with gradually increasing salinity. Studies in terrestrial caves have found that temperatures found within caves are relatively constant, approximating the mean annual temperature of the region, the same phenomenon holds true in aquatic caves where water temperatures at depth mirrors the average annual surface

temperatures (Barr, 1967; Humphreys, 1999). The deep water associated with Cherokee Road Extension Blue Hole (24.32°C) as well as the adjacent blue holes investigated during the expedition has temperatures close to the average annual surface temperature in Abaco. It was evident from the complex temperature plots (Figure 2.8a-d) that other processes are going on within Cherokee Road Extension Blue Hole and additional conservative mixing plots were created, looking at other water quality parameters such as hydrogen ion concentration and DO as a function of salinity (Figures 2.9a and 2.10a). There is a paucity of data on anchialine systems and how subterranean circulation, meteoric input, and temporal changes affect the overall physico-chemical profiles. Understanding these profiles in Cherokee Road Extension blue hole will provide an understanding of the biogeochemical processes occurring and how they relate to overall function of the bacterial mats present along the cave walls below the halocline.

During the March 2006 expedition to Abaco Island, divers investigated six inland blue holes, taking water quality measurements at each site. Interestingly enough, not all of the visited Abaco Island inland blue holes share the same water quality profiles as is typically seen in areas with numerous anchialine caves. Similar hydrological parameters to that of Cherokee Road Extension Blue Hole are present in sites including Treasure Cay Blue Hole and Water Station No. 1 (unpublished data) in which cooler water temperatures occur in entrance pools, with warming trends at depth and a well-defined H_2S layer.

These three inland blue holes also have similar geology, noticeable dissolutional undercutting and collapse, depths greater than 30 m, and little to no horizontal passage development. The remaining three inland blue holes investigated during the expedition, Dan's Cave, Ralph's Sink and Sawmill Sink, have extensive horizontal passageways and exhibit a cooling trend at depth (Botosaneanu and Iliffe, 2006). Differences in measured variables between islands or large limestone expanses are not uncommon and are documented elsewhere where anchialine caves are present.

Thermal anomalies

Anchialine caves from the Yucatán Peninsula also show varying hydrology. Stoessell *et al*., (2002) has documented several cenotes along the Caribbean coast of the Yucatán Peninsula with positive thermal anomalies associated at and below the halocline. Sulfate reduction at the halocline in deep sinkholes such as Cenote Angelita may be responsible for the formation of such thermal abnormalities (Stoessell, 2002), however, with an active deep water circulation found throughout the platform, sulfate reduction should only account for a fraction of a degree of increase. Sulfate reduction is induced when organic matter falling into these sinkholes interacts with the sulfate-rich intruding seawater below the halocline, causing hydrogen sulfide production, lowered pH values, increased alkalinity, and greater potential for limestone dissolution (Stoessell, 1992; Stoessell *et al*., 2002). Microbially mediated sulfate reduction

(e.g., *Desulfovibrio* sp.) has been found to occur in the lower portions of the halocline and in the underlying seawater. Conversely, sulfide oxidation may occur by means of anaerobic photosynthetic purple and green bacteria within lighted zones. Both processes lower pH below 6.9, enhance limestone dissolution, and are capable of forming a reddish-opaque boundary (Stoessell, 1995). Thermal anomalies present in Cenote Angelita and other cenotes along the Caribbean coast of Yucatán are a combination of both oxidation of aqueous sulfide in lighted zones and exothermic reduction reactions at depth (Stoessell *et al*., 2002).

Cherokee Road Extension Blue Hole is one of four known blue holes investigated on Abaco Island with strong H_2S layers and pH values below 6.8 at depth (Table 2.1). Cherokee Road Extension Blue Hole also resembles Yucatán's Cenote Angelita in depth and size and both contain hydrogen sulfide at the halocline and anaerobic seawater beneath. Both of these anchialine systems have an open air entrance pool, allowing organic debris to freely enter. Additional similarities include thick H_2S layers, and bacterial mats growing on the walls and overhangs below the halocline.

Cherokee Road Extension Blue Hole is of particular interest in that the temperature profiles are reversed (Figure 2.11) when compared to neighboring blue holes with extensive horizontal passages. Conservative mixing diagrams from nearby Dan's Cave (Figure 2.12a) at first glance looks as if there is an overall warming affect. Upon closer investigation (Figure 2.12b-d), a clearly

defined warming trend only at the halocline and both the fresh water and saltwater layers indicate a cooling shift. The temperature sink in the saltwater layer provides further evidence towards deep water circulation deep within the platform. As was noted, Cherokee Road Extension Blue Hole as well as other similar blue holes in the surrounding area has a slight increase in temperature at depth with cooler surface pool waters. The reversal of temperatures between the surface pool and waters at depth may be controlled by one of three mechanisms known to alter temperatures in anchialine systems in Australia and the Yucatán Peninsula.

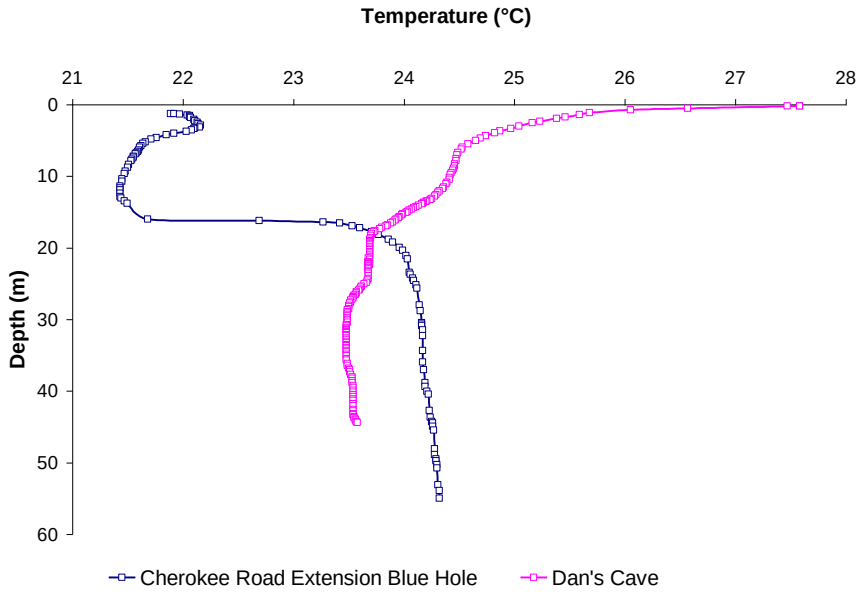


Figure 2.11 Vertical profile of temperature in Cherokee Road Extension Blue Hole and Dan's Cave showing differences in temperature at both surface and at depth. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and maximum water depth.

Water temperature in the surface pool of Bundera Sinkhole in Australia is lower than those at depth and is most likely due to evaporative cooling (Humphreys, 1999). Evaporative cooling could explain cool surface pool temperatures at Cherokee Road Extension Blue Hole. However, several entrance pools of blue holes nearby Cherokee Road Extension have higher temperatures in surface pools than that at depth (Table 2.1), which suggests evaporative cooling is not contributing towards decreased surface pool temperatures within Cherokee Road Extension Blue Hole. Evaporative cooling, while not unheard of from tropical regions, is usually confined to arid areas (Whitaker and Smart, 1993). Conservative mixing plots of measured water quality parameters (Figures 2.8 - 2.10) may provide a further insight into the processes occurring within Cherokee Road Extension Blue Hole such as bacterial respiration, microbial corrosion, and geothermal gradients.

Table 2.1 Temperature comparisons between inland blue holes on Abaco Island, Bahamas.

System Name	Surface temperature °C	Deep temperature °C/m	Lowest temperature °C/m	Highest temperature °C/m
Cherokee Road Extension§	21.89	24.32/54.95	21.43/11.90†	-
Treasure Cay§	23.66	24.68/41.68	22.66/14.88†	26.43/17.67‡
Water Station No.1§	23.85	24.63/34.31	21.53/10.40†	26.07/16.37‡
Dan's Cave	27.58	23.58/44.41	23.48/35.71	-
Sawmill Sink§	30.59	23.59/51.07	-	-
Ralph's Sink	25.51	23.80/25.63	-	-

§ – Strong H_2S layers with low pH values at depth
† – Denotes the top of the halocline
‡ – Denotes the bottom of the halocline

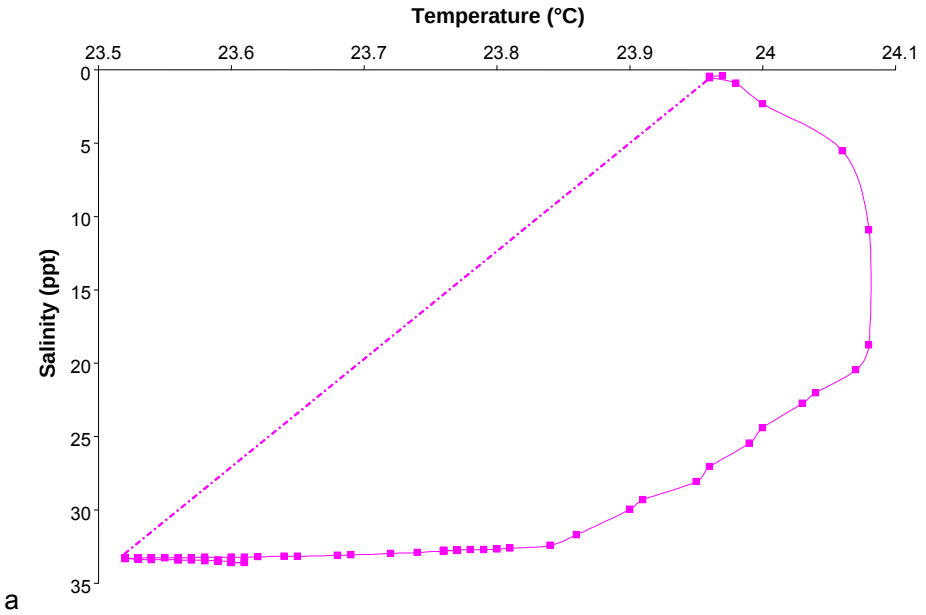
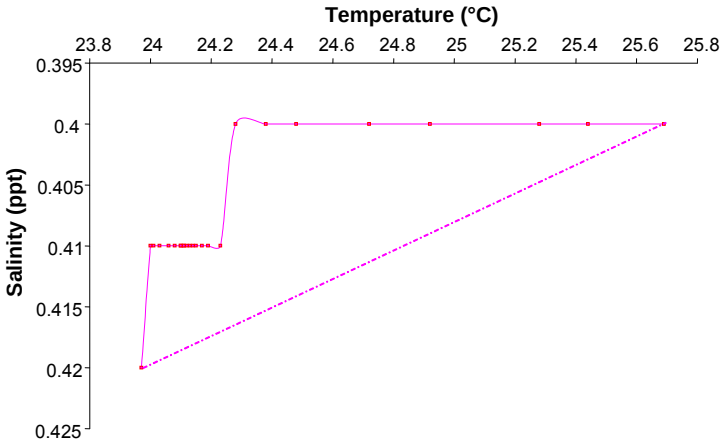


Figure 2.12 Salinity plots of temperature from Dan's Cave. Dashed lines between salinity and end members represent conservative mixing hypothesis. a) Mixing diagram through all three water masses. Plots (b-d) show mixing diagrams from each of the different water masses present: b) freshwater layer, c) mixing zone or halocline, d) saltwater layer. Individual measurements, indicated by □ symbol, were taken at 2 second intervals between the surface and maximum water depth.



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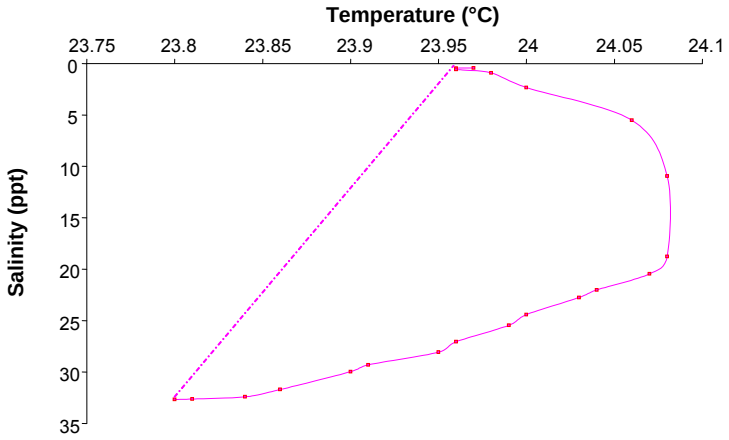

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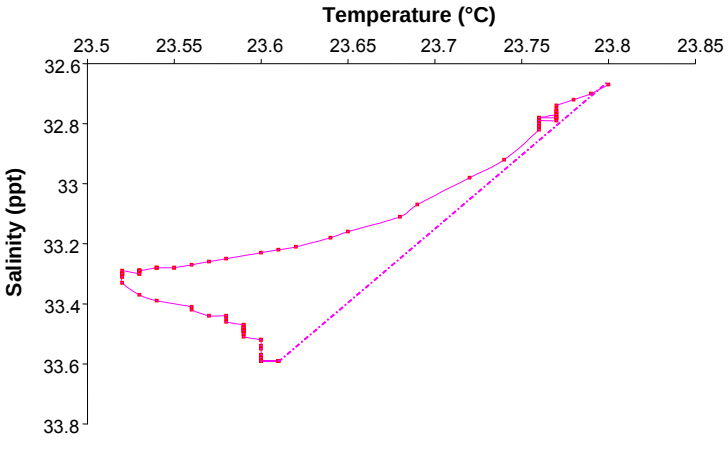

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Figure 2.12 Continued.

bacterial respiration may be responsible for thermal anomalies present in three coastal sinkholes on the Yucatán Peninsula (Stoessell *et al*., 2002).

Photosynthetic oxidation of sulfide to sulfate by anaerobic green and purple bacteria or anaerobic bacterial sulfate reduction may provide enough exothermic heat to cause a distinct rise in water temperature across the halocline as seen in Figure 2.8c in Cherokee Road Extension Blue Hole. Conservative mixing plots for temperature (Figure 2.8a) show that temperatures found throughout Cherokee Road Extension Blue Hole exhibit non-conservative mixing and convex upwards. The convexity of the salinity versus temperature plot suggests that water within the Cherokee Road Extension Blue Hole has warmed since entering suggesting that the cave is a heat source. Similar temperature versus salinity plots by Pohlman *et al*., (1997) and Humphreys (1999) illustrate that anchialine cave waters do not behave conservatively and act as either a net heat source or sink. Additional evidence (see Chapter III) suggests that endothermic reactions through microbial induced corrosion of limestone impurities may be responsible for thermal anomalies present at Cherokee Road Extension Blue Hole.

Microbial mediated corrosion

Many microorganisms can play a part in corrosion processes of metallic and non-metallic materials by which properties are altered and electrons are removed (Hamilton, 1985). The limestone walls of Cherokee Road Extension

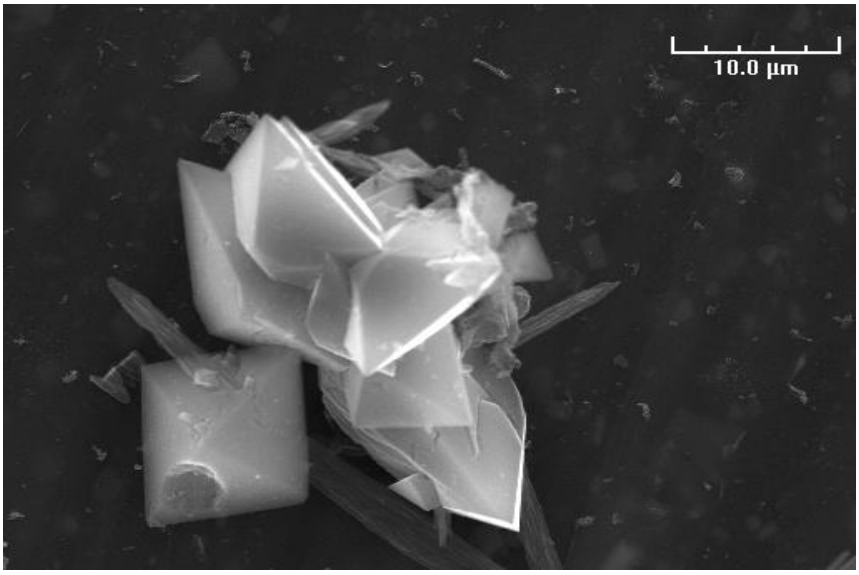

Figure 2.13 Scanning electron microscope image (2500 x 30kv) of pyramidal crystal inclusions from bacterial mat samples removed from Cherokee Road Extension Blue Hole. Scanning electron microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

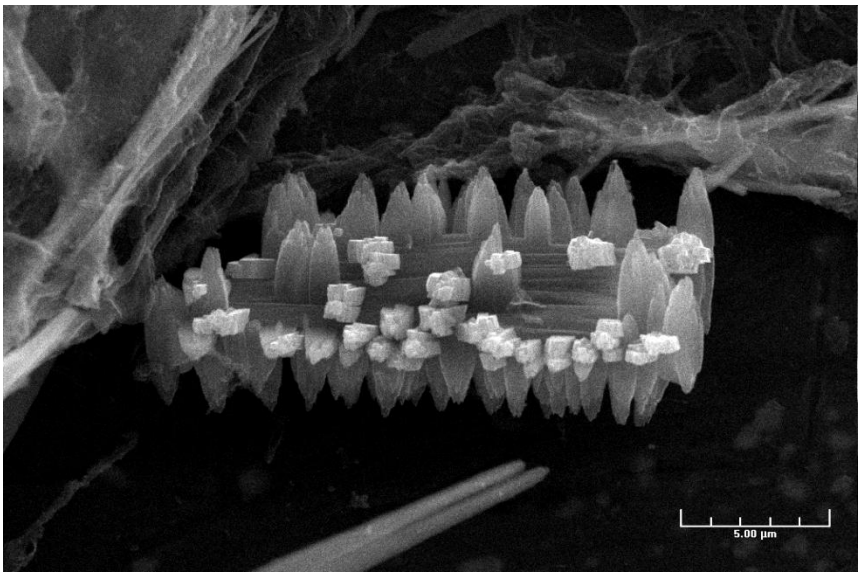

Figure 2.14 Scanning electron microscope image (4500 x 15kv) of crystal inclusions from bacterial mat samples removed from Cherokee Road Extension Blue Hole. Scanning electron microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

Blue Hole likely contain trace impurities such as magnesium carbonate, silica, alumina, iron, sulfur, and other minerals that are capable of undergoing microbial corrosion. The chief suspects in these bacterial mediated processes are those associated with the sulfur cycle, which include the sulfur-oxidizers and sulfate-reducers (Hamilton, 1985; Iverson, 1987). Corrosion induced by sulfate reducers typically occurs beneath microbial mats where oxygen is absent and conditions are less favorable for aerobic microbes (Hamilton, 1985). Cherokee Road Extension Blue Hole has several centimeter thick bacterial mats along the walls of the cave below the halocline. Clusters of crystal inclusions have been found at random throughout the microbial mat by SEM imaging suggesting that microorganisms may be the terminal link in the biodegradation of impurities found within the limestone (Figures 2.13 and 2.14).

Microbial induced corrosion involves the basic electrochemical mechanisms whereby electrons are removed from either oxygen or hydrogen ions (Iverson, 1987). As these electrons are removed, exothermic reactions produce heat energy, which is released into the surrounding water. One form of microbial induced corrosion is that present in Cherokee Road Extension Blue Hole is that of biogenic sulfide corrosion. This bacterial mediated process reduces sulfites from inorganic matter or from elemental sulfur producing H_2S. Wispy H_2S clouds are present at and slightly below the halocline. The pH of Cherokee Road Extension Blue Hole below the halocline is that of a weak acid, dropping as low as 6.28 and then gradually increasing at depth to 6.5. Chemical

analysis along with SEM imaging of the bacterial mat found along the cave walls (see Chapter III) suggests that within the microbial mat community, pH levels might range between pH 3-4. Chemical composition of crystal inclusions imbedded within the mat matrix requires low pH environments for precipitation of crystals. With such low pH levels occurring within the mat community, exothermic reactions occurring when acid from within the mat mixed with surrounding cave water might produce enough thermal energy to contribute to thermal abnormalities within Cherokee Road Extension Blue Hole.

Stoessell *et al*., (2002) proposed an additional theory that might elucidate the thermal anomalies found in Cherokee Road Extension Blue Hole. Localized seawater convection cells known from Floridian and possibly Yucatán aquifers (Stoessell *et al*., 2002; Reed *et al*., 2005) may be present in Bahamian aquifers. Porous groundwater traveling through deep karstic conduits could be heated by geothermal gradients and thus explain the thermal anomalies seen in Cherokee Road Extension and neighboring blue holes. However, since the Caribbean basins are stable and unaffected by Cenozoic seafloor spreading (Epp *et al*., 1970), it is unlikely that geothermal gradients would be responsible for increased temperatures on Abaco Island. If geothermal gradients were a plausible explanation for increased temperatures in Cherokee Road Extension Blue Hole, then all surrounding blue holes should show similar temperature parameters. All of the inland blue holes which show an increase in deep water temperatures are scattered throughout the island and located in proximity to those which show no

thermal anomalies. Since neither of the neighboring blue holes show elevated temperatures similar to those found within Cherokee Road Extension Blue Hole, it is doubtful that heat flow caused by geothermal gradients is responsible for the increase in temperature observed at depth in any of the sites with elevated temperatures.

Hydrogen ion production at the halocline supports non-conservative mixing when plotting the negative log of hydrogen ion concentration against salinity. The convexity of the graph suggests a source of hydrogen ions is present, similar to patterns seen in other reducing environments (Kemp, 1989). Similar non-conservative mixing patterns for hydrogen ion concentration is present in cenotes of Yucatán and blue holes of the Bahamas.

With apparent absence of oxygen occurring at depth below the halocline, the anaerobiosis at Cherokee Road Extension Blue Hole is similar and can be compared to mechanisms occurring below the uppermost few millimeters of aquatic sediments (Humphreys, 1999). Sulfide can be toxic to aerobic organisms at micromolar concentrations; in particular, crustaceans are highly sensitive to anoxia and sulfide (Fenchel and Finlay, 1995). However, the invertebrate fauna associated with Cherokee Road Extension Blue Hole does not avoid areas of low DO. Nerillid worms and three types of crustaceans: stygiomysids, ostracods, and numerous copepods, have been collected at or slightly below the halocline in Cherokee Road Extension Blue Hole. Cenote Angelita and Cenote Bacteria, both in the Yucatán Peninsula, host similar

stygobitic crustaceans in addition to amphipods, isopods, and thermosbaenaceans. The means by which these crustaceans and other invertebrates survive in such extreme environments is highly specialized and the product of millions of years of evolution. The detoxification mechanism that crustaceans use to survive in these extreme environments remains to be identified but may be similar to burrowing invertebrates such as nematodes, which assimilate with symbiotic sulfate reducers (Fenchel and Finlay, 1995; Humphreys, 1999). Hüppop (2005) comments on the phenomenon of a discontinuity in oxygen-uptake (oxyregulation) and classifies the adaptive abilities organisms possess to cope with the variable oxygen conditions. Oxyregulation within certain invertebrate stygiobites can be divided into two categories, oxyregulators or oxyconformers, based on their coping abilities to the variable oxygen regimes (Hüppop, 2005). Oxygen regimes in anchialine caves are known to range from well oxygenated waters to anoxic, but precise measurements are problematic (Sket, 1996; Humphreys, 1999). Tyson and Pearson (1991) subdivide environmental oxygen regimes into four categories: 1) oxic (10.64-2.66 mg/L), 2) dysoxic (2.66-0.266 mg/L), 3) suboxic (0.266-0.0 mg/L), and 4) anoxic (0.0 mg/L). The water associated with Cherokee Road Extension Blue Hole covers the entire range of the oxygen regimes, and the stygobites within are just as well adapted to cope with low DO as those found in the Yucatán.

Deep water circulation

Given the analytical limitations of the various instrument probes (Appendix I), it is evident that further and prolonged investigation is needed to precisely identify the mechanisms driving the physico-chemical profiles associated with Cherokee Road Extension Blue Hole. Nevertheless, the data collected is in accordance with Humphreys (1999) and Seymour *et al*., (2007) hypotheses regarding Bundara Sinkhole. Their studies indicate that meromictic anchialine systems have a minimum of two distinct microbial communities; one of photosynthesis by aerobic cyanobacteria and algae in the entrance pool driving oxidation and providing abundant organic material, and one of a lower anaerobic community. Circulation of oxygen rich waters at Cherokee Road Extension Blue Hole appears reduced across the halocline. Anaerobic microbes, such as those found in dense gelatinous mats aggregating along the walls below the halocline thrive in lower oxygen states, aiding in maintaining the water mass below the halocline in a chemically reducing state. Degradation of organic matter produced by eutrophic organisms in the entrance pools and the surrounding forest enhances production of H_2S, an important compound in reducing environments (Pohlman *et al*., 1997; 2000; Humphreys 1999).

Circulation within anchialine caves, especially those farther from the coastline (inland blue holes), is generally diminished and most certainly plays an important role towards maintaining a reducing environment (Humphreys, 1999). However, a certain level of circulation does occur given the nature of the

carbonate platforms and the highly porous nature of the limestone. Evidence of circulation in the form of noticeable water currents is not present in Cherokee Road Extension Blue Hole. However, daily tidal oscillations are evident from cyclic changes in the surface water level. Measurements of groundwater temperatures can be used as an indicator of circulation and provide information concerning the source of water within carbonate platforms (Whitaker and Smart 1993). A one-sample t-test (95% confidence interval) was calculated to determine if water temperatures at depth were significantly different from that of the average air temperature. Since three of the blue holes investigated have either increased bacterial presence or reducing environments with little to no horizontal passageways, they were initially excluded during the first statistical analysis. Of the three remaining blue holes with extensive horizontal passages (Dan's Cave, Sawmill Sink, Ralph's Sink), statistical results showed that bottom temperatures of these blue holes during the expedition to Abaco Island (Figure 2.15) are significantly cooler than the 2006-2008 annual air temperature of Rocky Cay, Elbow Cay, Abaco Island (25.5°C per IABACOEL1 Weather Station). The same statistical analysis was applied including all six blue holes investigated during the expedition (Figure 2.15) and showed that the temperatures were significantly lower than the 2006-2008 average annual air temperature for the area. Temperatures at depth in anchialine blue holes should mirror the mean annual air temperature in a given area, as is the case with air temperature in the deep interior of dry caves. Of the six inland blue holes

surveyed on Abaco during the March 2006 expedition, each had varying temperatures at depth, and all were lower than the mean annual air temperature (Figure 2.15). These profiles and statistical analysis suggest a deep-water circulation and origin of the water contained within.

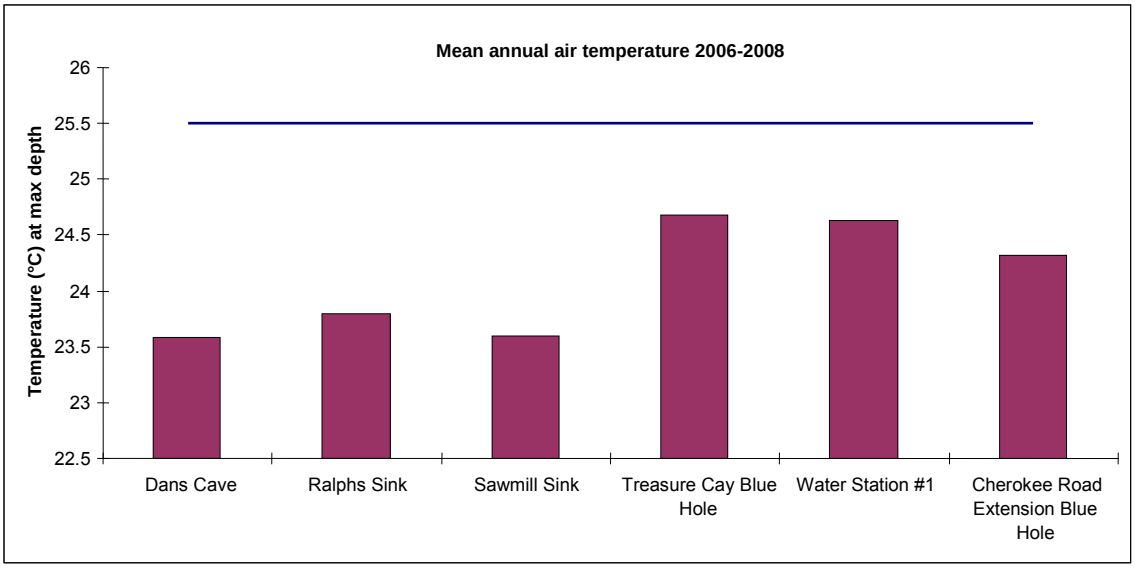

Figure 2.15 Deep water temperatures of all blue holes sampled during Abaco Island expedition. Blue line represents the average annual air temperature at Rocky Cay, Elbow Cay, Abaco Island weather station IABACOEL1 (2006-2008).

A common misconception noted by Cooper (1959) assumes salt water in coastal aquifer is static. In the presence of a zone of diffusion at the halocline, salt water is not static but in a perpetual cycle from the sea to the zone of diffusion and back. If a static state was to occur in the carbonate banks of the Bahamas, geothermal heat flux (1.0°C/40 m for adjacent Florida peninsula) would exist and a general warming trend would be detected at depth in inland

blue holes (Whitaker and Smart, 1993). Our observations (Figure 2.15) as well as those by Whitaker and Smart (1990 and 1993) suggest a cooling effect rather than a warming effect. Additional sonde data (unpublished data) gathered from Sawmill Sink shows a recovery of DO below a thick H_2S. No circulation of surface water penetrates the H_2S layer in Sawmill Sink, which further contributes to the idea behind deepwater circulation within carbonate platforms. The concave shape of the salinity-oxygen curve (Figure 2.10a) suggests that there is a production of DO present in Cherokee Road Extension Blue Hole however further investigation of the individual water masses (Figure 2.10b-d) indicate that the freshwater and saltwater masses act as a sink for DO but across the halocline, oxygen levels are sustained through horizontal flow. The rapid sink of DO within the saltwater layer is due to the highly reducing nature of the environment. Sawmill Sink, a nearby inland blue hole shows a recovery of DO below the chemocline. Salinity-oxygen curves for Sawmill Sink (unpublished data) suggest a source of oxygen present in the marine layer. Circulation of seawater within the carbonate platform both from horizontal flow and endo-upwelling provides the mechanism to introduce trace amounts of DO from the surrounding ocean depths into blue hole systems within the Bahamas and other carbonate areas.

Whitaker and Smart (1997) have proposed circulation of cold ocean waters deep within carbonate platforms. The data collected during the Abaco expedition indicates that similar phenomena may occur within the Little Bahama

Bank platform. Additional water parameter measurements of waters discharging from oceanic blue holes along the platform margins are needed for conclusive evidence of this circulation mechanism. Water parameter data in North Andros Island reported by Whitaker and Smart (1990, 1993) suggests that circulation of cold ocean water from depths greater than 200 m upward into the platform is sufficient to reverse the geothermal gradient effect and reduce temperatures at depth in blue holes below the mean annual temperature. Large-scale circulation of saline groundwater beneath North Andros Island does occur and similar circulation should be found on surrounding island banks. The circulation, if identified on Abaco Island, would comprise of saline waters from both the ocean basins and banks driven by a series of complex flow mechanisms involving groundwater temperature, sea surface elevation, and both vertical and horizontal salinity gradients (Whitaker and Smart, 1993).

The physico-chemical environment of anchialine systems like Cherokee Road Extension Blue Hole is complex and poorly understood and the measured parameters discussed provide only a glance and the true complexity of the water column. The complexity of these systems lies within the biogeochemical processes and vertical stratification occurring throughout the cave. Anchialine systems are delicate systems, capable of being significantly altered by human presence. For example, bubbles associated with open circuit SCUBA diving disrupt the vertical stratification of the water column and add oxygen to anoxic or at least hypoxic environments. Humphreys (1999) concluded that open circuit

SCUBA has a profound and generally negative effect on the anchialine system and the organisms inhabiting them. The implementation of closed circuit rebreathers (CCR) which do not release exhaust bubbles drastically reduces the diver's impact to these delicate environments. Continued research in anchialine systems found throughout the world will provide a better understanding into the fundamental biogeochemical processes driving such extreme and remarkable environments.

CHAPTER III

16 RIBOSOMAL RNA DIVERSITY OF AN ANCHIAILINE CAVE BACTERIAL MAT: CHEROKEE ROAD EXTENSION BLUE HOLE

Introduction

Anchialine caves host phylogenetically and biogeographically unique assemblages of stygobitic organisms. Although cavernicolous fauna were once thought of as exceptional accidents, nothing could be further from the truth (Vandal, 1965). Recognition that these ecosystems are of significant micro- and macro- biological significance has only occurred over the past two to three decades as the development of sophisticated diving technology has provided means to access these once foreign and inaccessible worlds (Pohlman *et al*., 2000). Many of the concepts presented below are based on terrestrial cave research, as a general understanding of anchialine systems has often times been theorized on terrestrial models. This research aims to elucidate the biogeochemical processes occurring in anchialine cave systems by assessing the genetic diversity of a microbial mat found in Cherokee Road Extension Blue Hole, Abaco Island, Bahamas.

Speleogenesis is the process whereby acidic groundwater dissolves limestone surfaces and creates voids. Traditionally, most caves form in the phreatic zone. If the water table lowers, vadose conditions are present and a once water filled passage now contains air, allowing for additional deposition of

speleogens and speleothems. There is however a few circumstances where spelogens can form underwater (e.g., scallops and dogtooth spar). These however require specific conditions to allow formations. Anchialine caves, blue holes and cenotes alike, are characteristically distinct from other caves in which there is typically very little vadose zone present, and unlike submarine caves which contain primarily only one distinct water mass, anchialine caves have vertically stratified water of marine origin under tidal influence (Pohlman *et al*., 1997; Humphreys, 1999; Iliffe, 2000; Pohlman *et al*., 2000; Seymour *et al*., 2007). There is however examples of anchialine caves scattered throughout the world which has both an extensive vadose and phreatic portion (e.g., Hatchet Bay Cave, Eleuthera and Green Bay Cave, Bermuda). The supply and source of particulate nutrients is however a common link between all subterranean environments; as well as the controlling factor surrounding distribution and growth of organisms (Airoldi *et al*., 1997).

Although the nature of organic inputs into anchialine systems is poorly understood, aquatic cave ecosystems are generally viewed as being allochthonous or detrital based, relying on material being transported in from the outside (Dickson, 1975; Pohlman *et al*., 1997). This “detrital mode” is controlled by heterotrophic microorganisms oxidizing organic carbon produced by photosynthetic means. It is suggested that as distance from the cave entrance increases, caves generally show a decline in biomass and diversity due to declining resources filtering off, sedimenting, and degrading (Fichez, 1990;

Airoldi *et al*., 1997). However, this paradigm does not hold true for many anchialine caves found in the Bahamas and Yucatán where an abundance of stygobitic fauna is present deep within the interior of the cave systems.

Pohlman *et al.* (1997; 2000) identified four point sources of organic matter which may account for the abundance of stygobitic fauna present within the deep interior of anchialine caves: 1) percolation of soil organics above the cave; 2) freshwater algae from entrance pools; 3) organic matter entrained by intruding seawater; and 4) chemoautotrophic bacteria in the interior of the cave. Pohlman's (1997) discoveries show that while several of the previously identified sources of organic matter may concurrently supply the Yucatán cenotes, there is however an overall heavy dependence on autochthonous chemoautotrophic inputs.

Subterranean ecosystems are one of Earth's major habitats that have received relatively little attention compared to others. Speleology, the study of all caves, has been argued to be too restrictive and an anthropomorphic ideology. Racoviță (1907) stated that speleology is truly the study of the subterranean world, which encompasses the sciences of geology, physical geography, and geophysics. It was not until the mid-nineteenth century that the science of biospeleology came into being. While early biospeleologists found that microbes inhabited terrestrial caves, little attention was directed towards water filled caves. As early as 1932, sulfur and iron bacteria (Ferrobacteriales, Thiobacteriales, Nitrobacteria) were thought to be present deep within terrestrial

caves studied throughout Europe; however their significance as producers was yet to be established (Vandal, 1965; Barr, 1967). With the absence of light and primary producers being limited to chemosynthetic autotrophs, the bacteria found in the subterranean world were often thought of as merely secondary degraders of materials transported into caves via air currents, human activities, insects, or water. Microbes were considered only as a source of nutrients for higher organisms (Caumartin, 1963; Barton and Jurado, 2007). Caumartin (1963) acknowledged that when water moves through the porous karstic terrain, numerous microorganisms are transported into subterranean habitats along with organic matter ensuring their survival. In highly karstified areas where surface streams are limiting, microbes and nutrients are carried into caves through meteroric events (Laiz *et al*., 1999; Engel and Northup, 2008). Such microbes must endure exposure to a carbonic acid solution, yielding an environment capable of sustaining chemically resistant microbes (Caumartin, 1963). Survival of microbes in this harsh subterranean environment is what characterizes the subterranean as distinctive from other environments. Little was published about cave-dwelling microorganisms until the 1990's. However, the few studies conducted were only able to isolate well-known soil bacteria and fungi, in addition to several protists embedded within cave clays and mud. It was within these sediments where the importance of microorganisms in caves was realized as they were found to play a nutritive role to several troglobites including the

blind amphipods *Bogidiella albertimagni* and *Niphargus* sp. (Vandal, 1965; Barr, 1967).

Subterranean microbes have been verified (from work by Caumartin, 1963) to be soil heterotrophs, as well as chemoorganotrophs, and fecal coliforms translocated into caves (Rusterholtz and Mallory, 1994; Engel and Northup, 2008). However, Engel and Northup (2008) pointed out that caves contain a wide range of physiochemical environments and parameters which dictate metabolic strategies by microorganisms in order to sustain life in such nutrient deficient ecosystems. With the vastness of the subsurface environment (>8 m depth for terrestrial settings and >10 cm in marine sediments per Whitman *et al.,* 1998), the extent of the microbial biomass is enormous and could exceed numbers from other components of the biosphere (Pace, 1997). With the lack of a continuous supply of nutrients, a secondary means of productivity devoid of allochthonous inputs and photosynthetically produced organic matter must be present. Several impurities are often associated with limestone deposition (e.g., Fe^{+2}, S^{0}, Mg^{+2}, Mn^{+2} and several other ferrous compounds) which provide a wide range of reduced metal ions for chemosynthetic microbes to synthesize (Caumartin, 1963; Engel and Northup, 2008). Barton and Jurado (2007) have found that subterranean systems can support microbial growth through three major routes of energy acquisition. Energy sources and nutrients can enter as: 1) atmospheric gasses (N_2 and CO_2) and organic molecules (including methylhalides and aromatic hydrocarbons); 2) soil derived aromatic compounds

percolating downwards with meteoric water; and 3) reduced metal ions (Mn(II) and Fe(II)) within the rock.

Karstic carbonate rocks are globally distributed and have been for over 3 billion years (most of Earth's history), becoming reservoirs for microbial communities and pathways of energy acquisition (Ford and Williams, 2007; Engel and Northup, 2008). The impact that microbes have on cave and karst environments is profound and can be seen today in caves scattered throughout the world. Carlsbad Caverns in New Mexico has unusual mineral deposits (U-loops and pool fingers) that are unexplainable by both geologic and inorganic processes (Barton and Jurado, 2007). Subsequent research found these structures indeed to be biogenic in origin, created from hanging microbial slimes and filaments acting as a nuclei for calcite precipitation (Spilde *et al*., 2005; Melim *et al*., 2009). In other instances, sulfidic caves have formed from microbial production of sulfuric acid, which reacts with the calcium carbonate to form gypsum and carbonic acid, thereby dissolving the host limestone rock and creating the cave from the inside out (Macalady *et al*., 2006). The most famous of these caves is Lechugilla Cave in New Mexico with over 184 km of known passageways. Several other more active sulfidic caves can be found around the globe and provide a valuable laboratory to study chemoautotrophically based cave ecosystems and microbial mediated biogeochemical processes analogous to early beginnings of life on Earth (Macalady *et al*., 2006; Barton and Jurado, 2007; Engel and Northup, 2008). Lower Kane Cave (Wyoming), Parker Cave

(Kentucky), Cesspool Cave (Virginia) and the recent discoveries in Movile Cave (Romania), Frasassi Caves (Italy), and Ayalon Cave (Israel) are of considerable interests to microbiologists. Within the groundwater systems and pools of the above mentioned caves, the microbial mats and biofilms contain a remarkable assemblage of aerobic and anaerobic sulfide-oxidizing lithoautotrophs (which fix inorganic carbon i.e., CO_2 using hydrogen sulfide as an energy source) as well as sulfate reducers, methanotrophs, and organoheterotrophic bacteria, many of these assemblages never seen before (Sarbu *et al*., 1996; Macalady *et al*., 2006; Por, 2007).

Each of the above mentioned cave systems have a significant hydrological components; however none of them are comparable to that of anchialine systems. Isotopic studies on marine cave systems in the Mediterranean and anchialine caves in the Caribbean (Cenote Maya Blue, Yucatán Peninsula) have demonstrated the presence of bacterial primary production by sulfide-oxidizing and nitrifying bacteria (Pohlman *et al*., 1997; 2000; Kinkle and Kane, 2000). The findings in each of these systems substantiate how understudied subterranean microbial ecology is in truly aquatic cave systems. Despite the importance of chemolithoautotrophy and its role in ecosystem functioning (Engel and Northup, 2008), there is a paucity of data on anchialine systems that documents the presence, distribution, diversity, and metabolic potential of the microbial communities. The microbial diversity and the wide array of ongoing geochemical processes is what makes anchialine systems

valuable as natural experiments, perfect analogs for both early terrestrial communities and that of stratified and sulfidic oceans present early in Earth's history (Macalady *et al*., 2006; Gonzalez *et al*., 2010).

Anchialine blue holes are extreme environments, which aside from the entrance pools, are lightless, geologically isolated, and energy-limited, receiving little if any surface derived nutrients (Poulson and Lavoie, 2000; Barton and Jurado, 2007). Unlike anchialine caves, terrestrial caves often times remain static receiving little seasonal or temporal variation (Barton and Jurado, 2007). However, conditions in anchialine blue holes found throughout the Bahamas are not static and fluctuate daily from tidal oscillations. Bahamian inland blue holes also are prone to endo-upwelling which constantly circulates water deep within the platform. Both of these mechanisms have the potential to distribute and introduce sources of organic nutrients (Cooper, 1959; Whitaker and Smart, 1997).

Anchialine caves in the tropics have had less destructive effects from glacial events on the overall lithology than those caves in temperate zones. Connectivity between the subterranean and the surface may increase over geologic time (thousands to millions of years) (Poulson and Lavoie, 2000). Increased carbonate dissolution in undisturbed limestone beds substantiate the claims made by Pohlman *et al*., (1997; 2000) that surface derived nutrients may enter anchialine ecosystems. Evidence supporting increased connectivity in tropical karstic areas can be seen in caves where physical-chemical profiling

reveals areas of horizontal flow below present day halo/chemoclines. These regions are caused by glacial eustatic events lowering present sea levels allowing for dissolution of limestone to occur as the halo/chemocline shifted. Sea level notches can be observed along the Bahamas coastline indicating glacial eustatic events. Similar horizontal notches are also present within inland blue holes. These areas usually show signs of heavy dissolution with brittle limestone.

Microbial diversity and function has long since been known in the carbon rich terrestrial environment. However, the vast majority of microbial diversity does not occupy energetically favorable environments; this has significant impact on biogeochemical processes in karstic terrains (Herman and Hubbard, 2002; Barton and Jurado, 2007; Engel and Northup, 2008). The importance of microbes in subterranean karst, especially that of anchialine caves, is underappreciated. Anchialine blue holes like many other geologic environments have redox stratified groundwater bounded by reactive rock surfaces which provide a wide assortment of energy sources, all of which potentially harbor characteristic microbial populations (Amend and Teske, 2005; Seymour *et al*., 2007; Engel and Northup, 2008). The fresh/brackish water layer in anchialine systems is oxic, dominated by aerobic respiration. A decrease in dissolved oxygen and redox potential occurs as depth increases, usually associated with denitrification and metal reduction (Amend and Teske, 2005). Sulfate reduction occurs at and below the density interface (halo/chemocline) in the presence of

seawater sulfate and microbial metabolism of organic matter. On rare occasions, autotrophic methanogenesis may occur in conjunction with extremely low redox potentials (Amend and Teske, 2005).

Inland blue holes of the Bahamas typically show redox stratification, many of which with sulfidic layers; yet the biogeochemical cycling is complex and poorly understood. Previous works on the microbiology of anchialine systems (cenotes and blue holes) were based on microscopy or culturing, both of which introduce biases since culturing practices are often impractical since replication of the cave environment is impossible and ~90-99% of microbes are unculturable (Engel and Northup, 2008). This study provides one of the few studies which utilizes 16s rRNA clone libraries to assess microbial mat diversity and its relationship to biogeochemical processes.

Considering the distribution and sensitive nature of anchialine systems, it is imperative that we gain a more concise understanding of the microbial diversity present in these anchialine blue hole systems, and to what extent the biogeochemical and ecological conditions have on controlling not only the microbial diversity, but that of the invertebrate and vertebrate diversity.

Methods

In March 2006, a two-week scientific cave diving expedition was conducted on Abaco Island, Bahamas as part of a National Science Foundation grant from the Biodiversity Surveys and Inventories Program (DEB-0315903)

surveying cave fauna of the Bahama Islands. One area of emphasis was placed on blue holes containing bacterial films.

Cherokee Road Extension Blue Hole is an inland blue hole located 20 km southwest from Marsh Harbour, on Abaco Island, Bahamas. Situated in a pine forest, Cherokee Road Extension Blue Hole is a water-filled, collapsed sinkhole with little known horizontal passageway, depths of 104 m, and a several centimeter thick gelatinous orange bacterial mats present along the walls just below the halocline (Figure 3.1).

Exploratory cave dives and sample collections complied with the cave diving regulations set forth by the International Association of Nitrox and Technical Divers (IANTD) and that of the Texas A&M University at Galveston Dive Control Board (TAMUG-DCB) under the auspices of the American Academy of Underwater Sciences (AAUS). Under these regulations, all cave diving conducted throughout this expedition adhered to the five primary rules of cave diving: 1) never exceed your training, 2) use of a continuous guideline at all times, 3) utilize the rule of thirds for gas management, 4) do not exceed the proposed dive plan or the safe operational depth of the breathing mixture, and 5) always carry three sources of light (Exley, 1977; Mckinnon, 1992).

All samples collected throughout the expedition were authorized in the form of a biological collection permit issued to Dr. Thomas Iliffe from the Department of Fisheries - Ministry of Agriculture, Fisheries, Nassau, Bahamas.

Figure 3.1 Rebreather diver Gregg Stanton (Wakulla Diving Center, FL) towing plankton net alongside concentrations of bacterial mat found in Cherokee Road Extension Blue Hole, Abaco, Bahamas. Photo courtesy of Tamara Thomsen, Diversions Scuba, WI.

Underwater samples were collected using pre-labeled, sterile, 50 mL conical screw cap tubes (USA Scientific 1500-1811), pre-filled with autoclaved milli-Q water to prevent implosion at depth. Sterile techniques were used at all times to prevent diver contamination. This included touching only the outside tube and cap, keeping exhaled bubbles away from specimens and tubes, and keeping hands out of collected material. Due to differences in water densities, an exchange of milli-Q water for salt water was observed at depth. To ensure complete removal of the milli-Q water before gathering specimens, each tube was shaken vigorously, exchanging any remaining milli-Q water for surrounding cave water. A horizontal scooping motion was used to gather bacterial mat samples into the tube. Once obtained, the tube number as well as the depth

and location within the cave (above or below halocline) were recorded on the diver's wrist slate. Tubes filled with bacterial mat samples were placed in separate collection pouches to prevent cross contamination or confusion of vials while underwater. Tami Thomsen (Diversions Scuba, WI) provided photo documentation of site characteristics and collection techniques, both above and below the water.

Upon completion of the dive, all samples were placed in a flexible, dark cooler containing with frozen ice packs or ice for transport back to an improvised lab within our hotel. All samples were stored in a refrigerator in the dark until processed (generally within one hour from returning from dive site). Processing the samples includes decanting cave water from the collected material and filling the tube with molecular grade ethanol (IBI Scientific, Peosta, IA). Vials were capped, sealed with parafilm, labeled, and placed in the hotel freezer (-20°C) until transport back to Texas A&M University at Galveston. Samples were packed with frozen ice packs during flight back to the Marine Biospeleology Lab and the Coastal Health and Estuarine Microbiology Lab located at Texas A&M University at Galveston, where upon arrival there were placed in a -20°C freezer for later molecular analysis.

Nucleic acid extraction and amplification

Genomic DNA obtained from environmental samples used a bead beating protocol (Appendix II) developed by John R. Spear (Colorado School of Mines,

pers. com). Fragments of the bacterial mat sample from 40.8 m water depth (approximately 3 mm^3 in volume) were transferred into 2 mL conical vials containing 1 mm glass beads (BioSpec Products, Inc.). 500 µL of extraction buffer containing NaCl, Tris (tris-hydroxymethyl-aminomethane) and EDTA (ethylenediaminetetraacetic acid) was added to the sample followed immediately by the addition of 200 µL SDS (sodium dodecyl sulfate). Material was incubated at 37°C followed by the addition of 500 µL phenol:chloroform (1:1) to further extract the DNA and remove organic contaminants. After centrifugation, the aqueous layer was removed and placed into a new vial where an additional 500 µL phenol: (1:1) was added and mixed. Nucleic acids were precipitated using 600 µL of cold isopropanol and 60 µL sodium acetate. Samples were suspended in 20 µL TE buffer (Tris and EDTA) to protect against degradation, and stored in -20°C for later PCR analysis.

DNA purity of extracted material was analyzed using a Beckman Multicell Module spectrophotometer. DNA absorbs UV light with an absorption peak at 260 nm, while proteins tend to absorb at 280 nm. A ratio of 260:280 between 1.7 and 2.0 is considered 'pure' DNA (Zhou *et al.,* 1996). If absorption occurs at 230 nm it indicates organic contamination in the DNA sample.

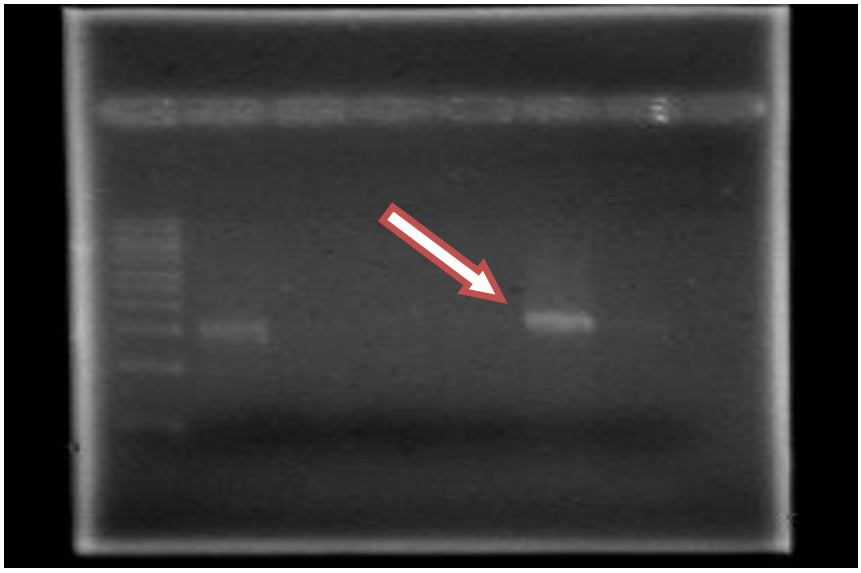

Figure 3.2 1% agarose gel showing PCR product (maroon arrow). Environmental DNA extracted from a microbial mat below the halocline in Cherokee Road Extension Blue Hole, Abaco Island, Bahamas.

Extracted DNA was cleaned using the WIZARD DNA Clean up system (Promega Corp., Madison, WI) to remove co-extracted contaminants that may inhibit polymer chain reaction (PCR). Nearly full length 16s rRNA isolates were amplified by PCR using an automated thermal cycler (Eppendorf, Germany) with bacterial specific forward and reverse primers [8*f* (*5'-AGA GTT TGA TCC TGG CTC AG-3'*) and 1492*r* (*5'-TAC GGY TAC CTT GTT ACG ACT T-3'*)] (Integrated DNA Technologies, Coralville, IA). The PCR reactions were 50 µL in volume and contained 0.5 µL of each primer (10 µmol), 1 µL dNTPs (10 mmol), 1 µL BSA, 5 µL PCR buffer (Roche, Switzerland), 0.5 µL Taq (Roche). Final volume (50 µL) of the PCR reaction was reached with the addition of PCR water. Between 1 µL to 5 µL of sample DNA (100 ng/µL) was added to the final reaction mixture. PCR run conditions were as follows: initial denaturation step at

94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 3 minutes, followed by a final extension at 72°C for 10 minutes. PCR products were checked using a 1% agarose gel with a 1KB ladder (Sigma-Aldrich Co., USA). Upon successful amplification (Figure 3.2), PCR products were cleaned using the Wizard PCR Preps DNA Purification System (Promega Corp., Madison, WI) and stored until cloning.

Ligation

Cleaned PCR product was ligated using the pGEM-T Easy Vector Systems (Promega Corp., Madison WI) in preparation for cloning. The ligation reactions were set up as follows: 5 µL 2x Rapid Ligation Buffer T4 DNA Ligase, 1 µL pGEM-T-Easy Vector (50ng), 2 µL – 3 µL cleaned PCR product (volume based on intensity of band on gel), 1 µL T4 DNA Ligase, and PCR water. These reactants were mixed and incubated overnight at 4°C to obtain a maximum number of transformants.

Cloning

Ligation reactions were transformed into circular plasmid cells and inserted into *Escherichia coli* JM109 cells (Figure 3.3). Reaction tubes were centrifuged and 2 µL was transferred into a sterile15 mL Falcon tube on ice. 50 µL of JM109 High Efficiency Competent Cells (Promega) were carefully transferred into each 15 mL sterile Falcon tube. The cells were then heat-

shocked for 50 seconds in a 42°C water bath and then immediately returned to ice for two minutes. 950 µL room temperature SOC medium (Bacto-tryptone, Bacto-yeast extract,1M NaCl, 1M KCl, 2M Mg^{2+}, 2M glucose) was added to each tube. Tubes were incubated for 1.5 hours at 37°C with shaking (150 rpm). 100 µL of each transformation culture was plated onto duplicate or triplicate LB/ampicillin/IPTG/X-Gal plates equilibrated to room temperature and incubated overnight at 37°C. Following incubation, pates were color screened (white colonies contained the vector insert and blue colonies were lacking). One hundred of the larger of the white colonies were transferred with sterile toothpicks onto fresh LB/ampicillin/IPTG/X-Gal plates equilibrated to room temperature and incubated overnight at 37°C. Following incubation, half of each of the 100 colonies were transferred with a sterile toothpick into 0.5 mL tubes containing 24 µL of 2x cracking buffer (5M NaOH, 0.5M EDTA, 10% SDS, 100% Glycerol, deionized water) which digests the competent cells and releases the plasmid containing the desired DNA fragment. DNA fragment size was measured by running a 1% agarose gel stained with ethidium bromide, and visualized under UV light. If the desired length was achieved, the remaining half of each colony was further incubated overnight at 37°C followed by transfer with a sterile toothpick onto fresh LB/ampicillin/IPTG/X-Gal plates equilibrated to room temperature. Selected colonies were transferred with a sterile flame loop to a 15 mL sterile Falcon tube containing 5 mL LB broth. Tubes were incubated overnight at 37°C with shaking (150 rpm). After centrifugation, the cell pellet

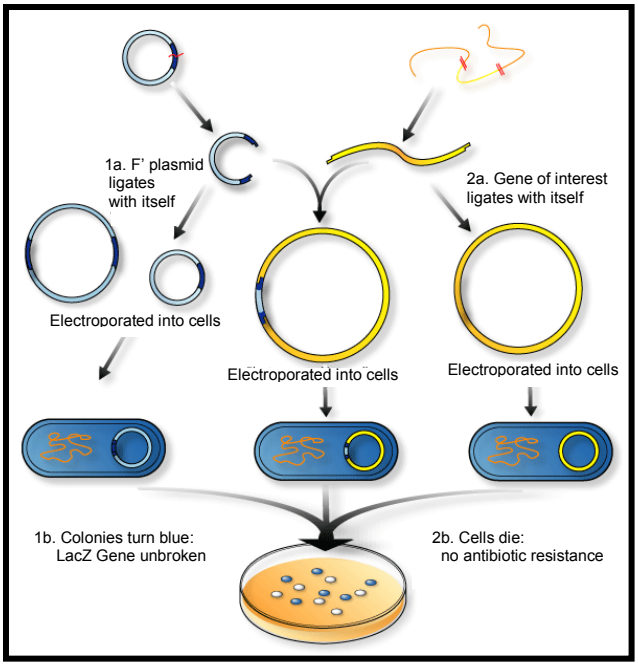

Figure 3.3 Diagram of cloning process (The Science Creative Quarterly http://www.scq.ubc.ca). The process where fragmented DNA is recombined and incorporated into the cells where replication occurs, creating daughter cells with newly generated plasmids is depicted.

was collected after decanting the supernatant, and stored at -20°C until plasmid extraction.

Clone libraries were constructed based on extraction of plasmid DNA. Plasmid extraction was completed using the E.Z.N.A. Plasmid Miniprep Kit I (Omega Bio-Tek, Inc., Doraville, GA). The extracted plasmid containing the desired DNA fragment was stored at -20°C for later analysis. PCR verified the extracted plasmids contained the 16s rRNA fragment and assured they were pure enough for downstream analyses.

ARDRA

Amplified rDNA restriction analysis (ARDRA) using two restriction endonucleases (*HaeIII* and *RsaI*) were used to screen the 16S rRNA gene diversity within the clone libraries. ARDRA is a genetic fingerprinting technique that is performed on PCR-generated rDNA fragments whereby endonucleases (restriction enzymes) cut the DNA fragments at specific restriction sites creating unique patterns for different bacteria. In order to increase phylogenetic resolution, *HaeIII* and *RsaI* restriction enzymes were used in conjunction since *HaeIII* has a high average number of restriction sites per taxon and *RsaI* is good at differentiation amongst diverse bacterial taxa (Moyer *et al*., 1996). The recognition sequences for *HaeIII* (5'…GG↓CC…3') and *RsaI* (5'…GT↓AC…3'). Resulting ARDRA patterns were separated on an 8% acrylamide gel (19:1, acrylamide / bis-acrylamide), and stained with ethidium bromide for visualization under UV light (Appendix III). Banding patterns of the ARDRA gels were analyzed using GelCompar II (Applied Maths, NV). The cluster analysis method used was the comparative numerical analysis with the unweighted pair group method using arithmetic averages (UPGMA). Based on this cluster analysis, one or more representatives of each ARDRA pattern group, representing a 16S rRNA phylotype or operational taxonomic unit (OTU) from all clone libraries were selected for sequencing.

DNA sequencing

The DNA Analysis Facility on Science Hill at Yale University performed all sequencing services (http://research.yale.edu/dna_analysis/). 16s rRNA 's were sequenced using an ABI 3730 capillary sequencer (Applied Biosystems, Inc., Foster City, CA) using vector specific primer M13*f*.

Phylogenetic analysis

All sequenced data was analyzed against 10,000+ previously aligned entries, as well as using other related entries, from GenBank using the ARB database software (February 2005 release). All sequences were checked for chimera's using Green Genes Chimera check with Bellerophon (version 3).

Neighbor joining trees were constructed using jukes cantor correction using the ARB database software. Only sequences having 1000+ base pairs were used to calculate the trees. Shorter sequences were added once the tree was calculated.

Shannon Weiner diversity indices were calculated according to Zar (1999). Sampling sufficiency and species richness within the clone library was assessed using rarefaction curves (Analytic Rarefaction 1.3; http://www.uga.edu/~strata/software/Software.html).

Coverage (Mullins *et al*. 1995) was calculated to evaluate the actual diversity represented by our clone libraries. Coverage was derived from the equation:

$$C = 1 - (n_1/N)$$

where, n_1 is the number of clones that occurred only once and N is the total number of clones examined. This value is conservative, but excludes variation introduced by PCR artifacts and heterogeneities in rRNA gene families (Mullins *et al*., 1995).

All cloned sequences were abbreviated as 'CRE-MAT' for Cherokee Road Extension Blue Hole mat. Nearly full-length 16s rRNA sequences generated in this study were deposited in GenBank.

Results

The bacterial mat in Cherokee Road Extension Blue Hole (observed by cave divers) initiated, and was at its thickest immediately below the halo/chemoline (25.6 m) and tapered with depth. Spongy orange mat was found attached to the limestone cave walls only on upper surfaces exposed to light and absent from the undersides of ledges and boulders. A total of 65 non-chimeric 16s rRNA sequences were obtained and used for construction of dendrograms. Appendix IV lists the entire diversity of sequenced clones as well as percent similarity to both cultured and uncultured bacteria previously reported in GenBank (http://www.ncbi.nlm.nih.gov).

The community structure of the 16S rRNA phylotypes obtained from Cherokee Road Extension Blue Hole is listed in Figures 3.4 - 3.7. A distinctive feature of this mat is the distribution of phylotypes across major subdivisions in

the bacterial domain. These blue hole phylotypes fall into divergent branches which include previously described groups, well defined by culture and or culture independent means. These branches include *Chlorobium* and Candidate Division OP8. A diagrammatic phylogenetic tree of cultured and cloned representatives from the bacterial group *Chlorobium* is present in Figure 3.4. The mat sample used for analysis shows that 14 clones have a strong affinity to *Chlorobium*, a photolithotrophic sulfur oxidizer. Twenty-one clones (Figure 3.5) show affinities to the newly discovered Candidate Division OP8 from an obsidian pool in Yellowstone National Park (Ley *et al*., 2006). A total of 22 clones are grouped amongst a lineage of bacteria, but do not align to any one particular group and may represent novel subdivisions with relative similarity to known bacterial groups *Defferibacter, Fibrobacteres, Verucomicrobium and Desulfacinum* (Figure 3.6), all which are capable of reducing sulfur, sulfate, and nitrate.

Bacterial clones from nearby blue hole, Sawmill Sink, located 20 km to the southwest (Macalady *et al*., *in prep.*), are compared with clones from Cherokee Road Extension Blue Hole. Affinities between clones from Cherokee Road Extension blue hole and those found in Sawmill Sink are presented in Figure 3.7.

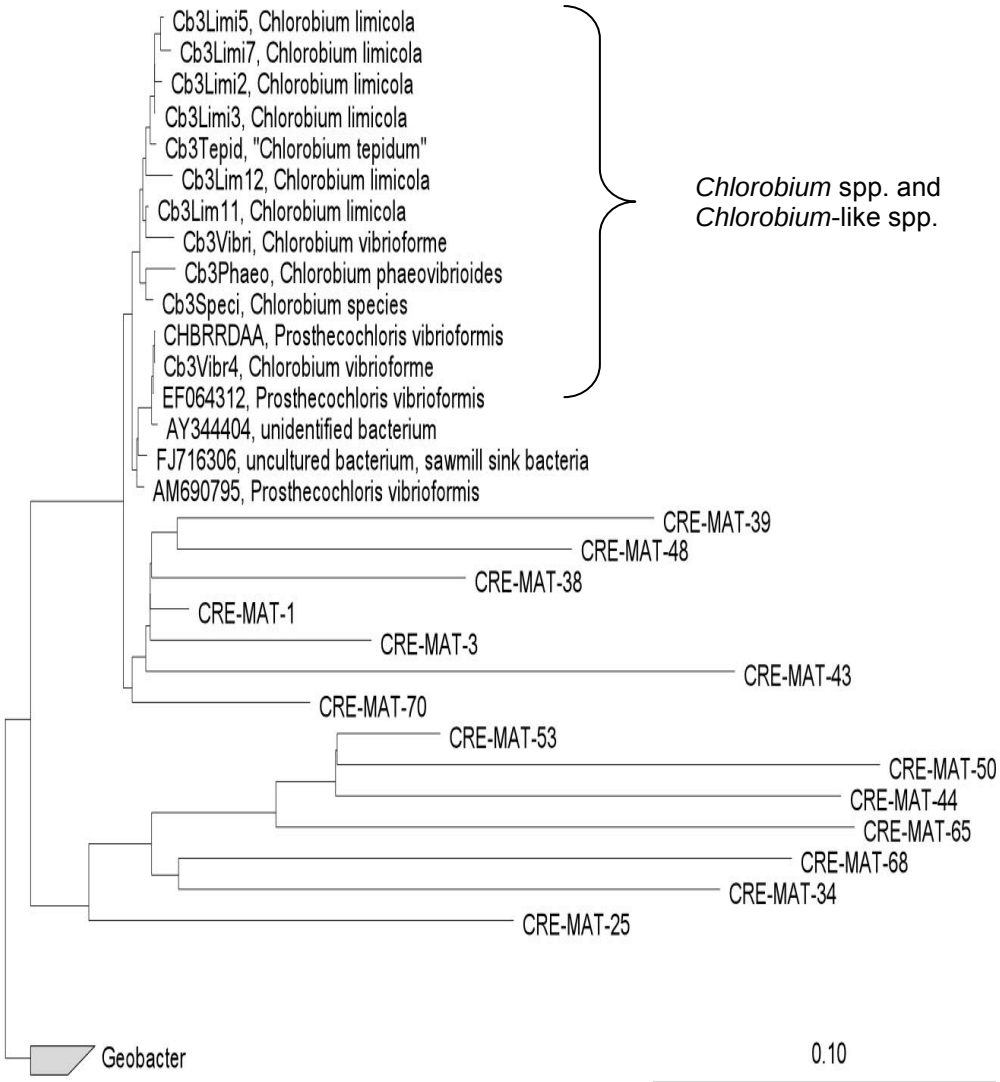

Figure 3.4 Cherokee Road Extension Blue Hole clones showing affinity to bacterial group *Chlorobium*. Neighbor joining tree using Geobacter as the outgroup. Scale bar represents 10% sequence divergence.

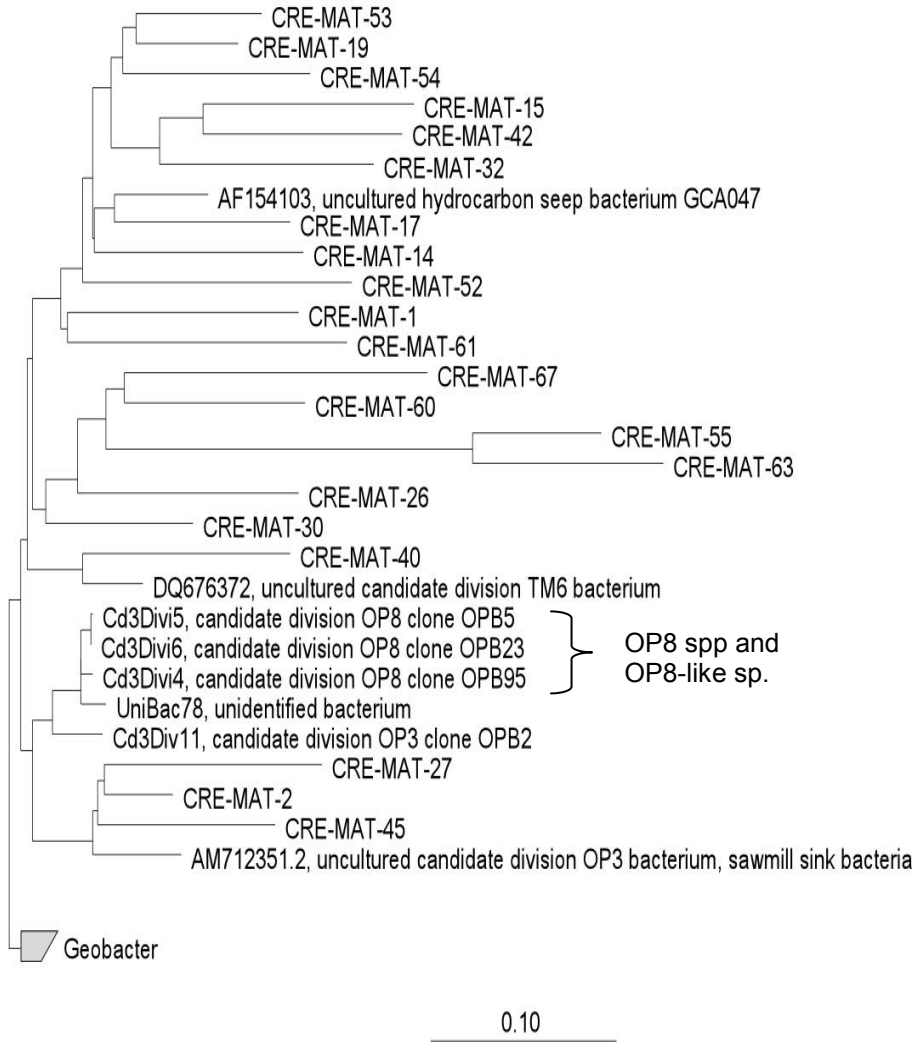

Figure 3.5 Cherokee Road Extension Blue Hole clones showing affinity to bacterial group Candidate Division OP8. Neighbor joining tree using Geobacter as the outgroup. Scale bar represents 10% sequence divergence.

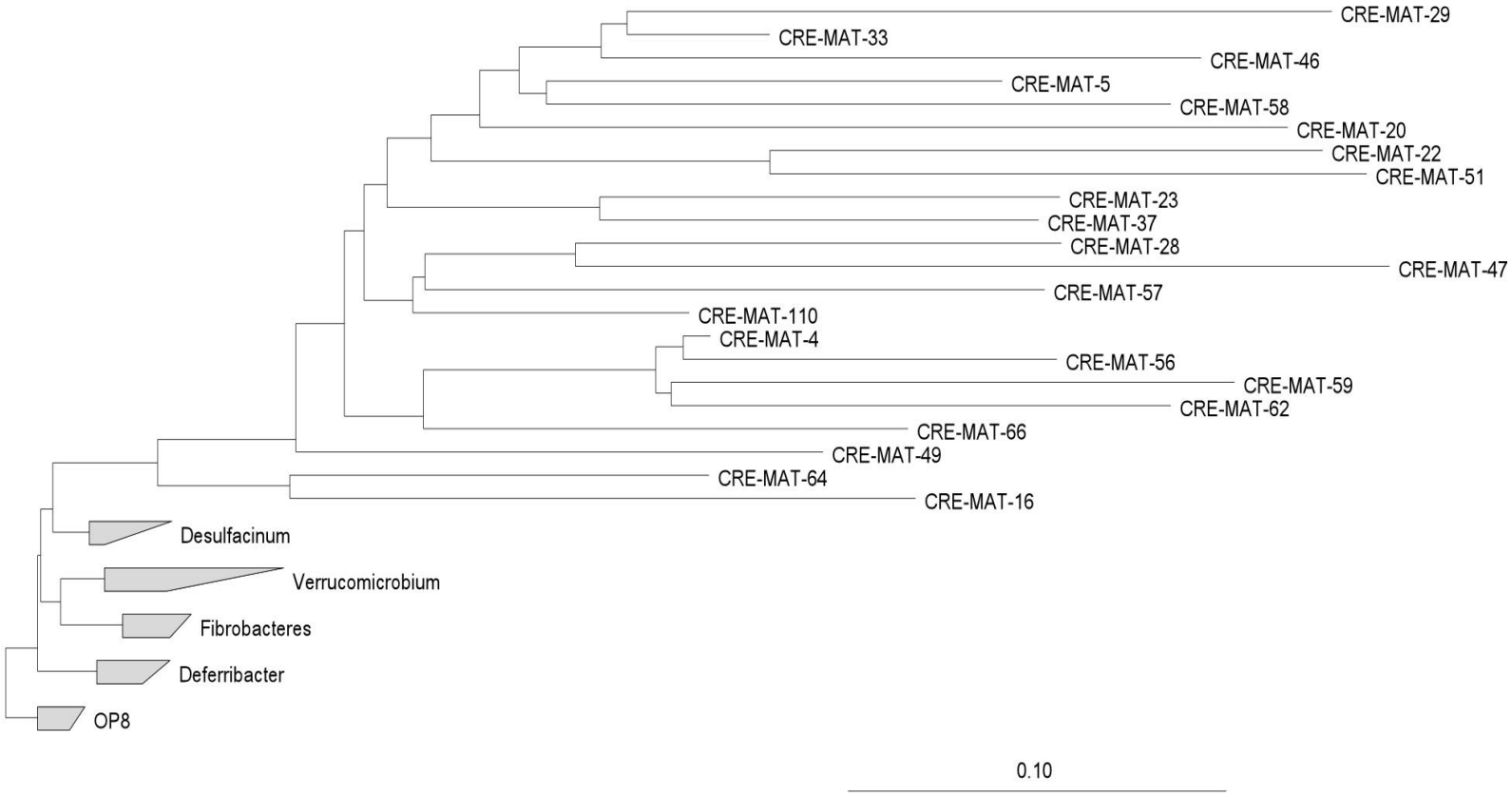

Figure 3.6 Cherokee Road Extension Blue Hole clones showing affinity to bacterial group Deferribacter and close affinities. Neighbor joining tree using OP8 as the outgroup. Scale bar represents 10% sequence divergence.

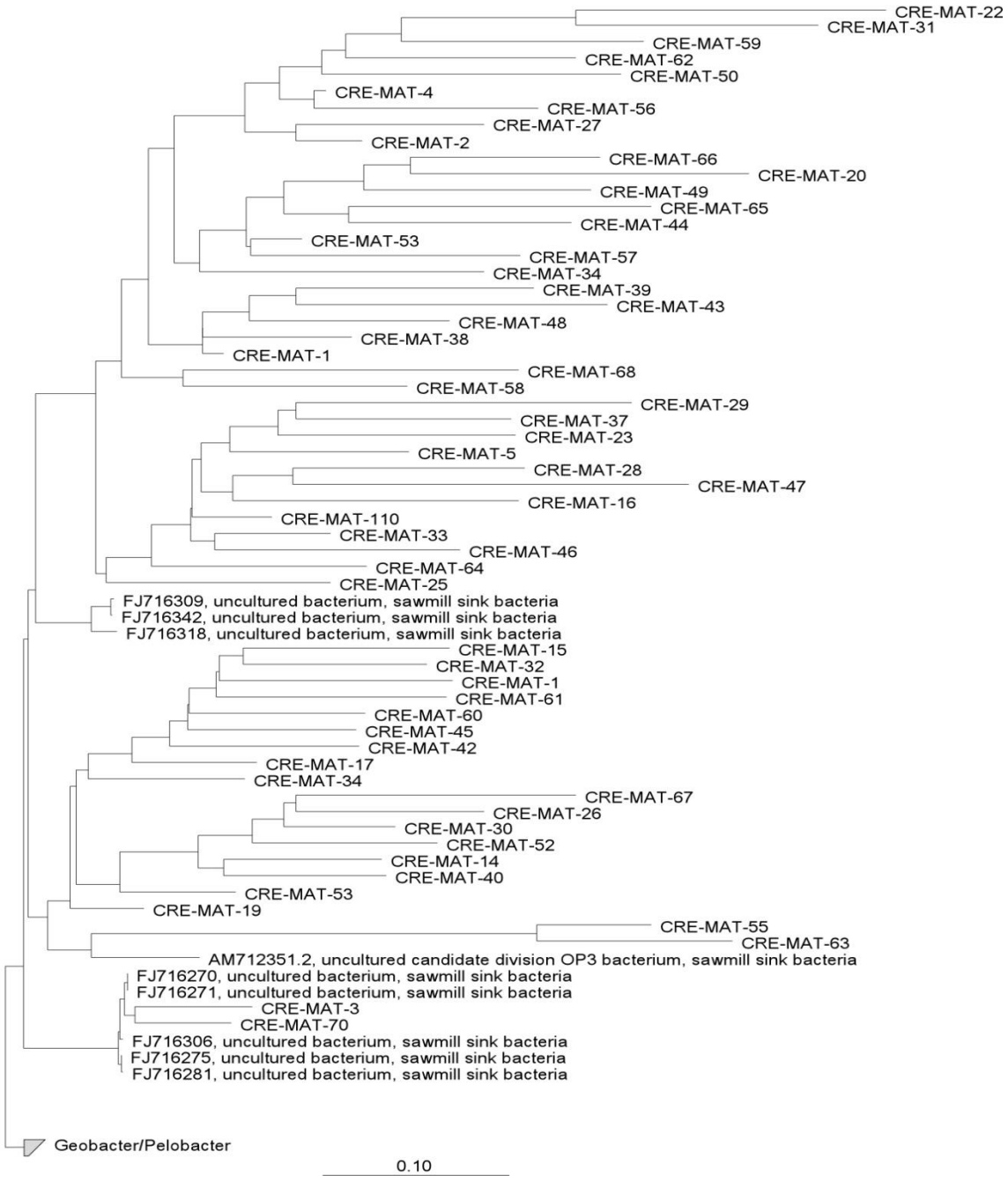

Figure 3.7 Comparison of clones from adjacent blue holes, Sawmill Sink and Cherokee Road Extension Blue Hole. Microbial communities from Sawmill Sink were analyzed by Dr. Jennifer Macalady, Penn State University. Geobacter/Pelobacter represents the outgroup. Scale bar represents 10% sequence divergence.

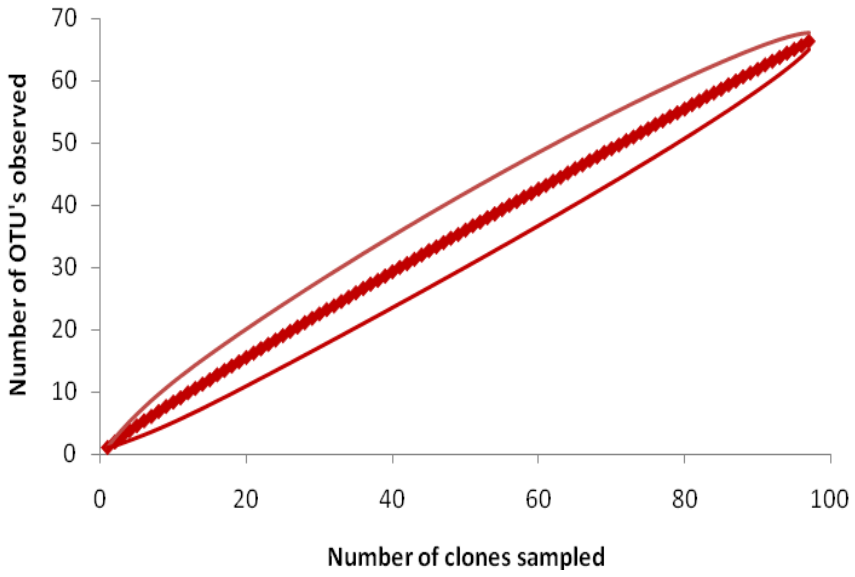

Figure 3.8 Rarefaction curves based on 16s rRNA clones from microbial mats present in Cherokee Road Extension Blue Hole, Abaco Island, Bahamas. Operational taxonomic units (OTU) are defined by 98% sequence similarity.

The rarefaction curve (Figure 3.8) calculated from the constructed clone library from Cherokee Road Extension Blue Holes has a steep upward slope, rather than plateauing, indicating the potential to detect additional bacterial phylotypes. Improved extraction methods, construction of additional clone libraries and the use of primer sets targeting specific groups should yield a more diverse subset of bacteria. Coverage calculations showed that only 35% of the diversity was sampled and according to Shannon Weiner diversity indices, where H'=4.205, the bacterial mat present in Cherokee Road Extension Blue Hole is considered complex with high diversity. Epifluorescence microscopic examination of extracted mat material using DAPI (4' 6'-diamindino-2-

phenylindole) that strongly binds to DNA did reveal some ‘thread-like’ structures in the mat that were still intact. However, it could not be determined if these structures were bacterial cells.

Discussion

This phylogenetic 16s rRNA analysis of the microbial mat present on the cave walls below the halocline in Cherokee Road Extension Blue Hole is one of the few investigations of bacterial genetic diversity from an anchialine blue hole. Results are characteristic of low energetic microbial mat systems, where several bacterial assemblages are present in a mutualistic consortium. Results only take into account the diversity of the microbial mat at 40.8 m during March 2006. In total, this phylogenetic analysis only provides a glance at the true complexity of the microbial mats present in Cherokee Road Extension Blue Hole.

The subterranean biosphere is more expansive and complex than previously thought and is evermore increasing as scientists probe further and deeper into the subterranean abyss. The diving technology needed to access anchialine environments has expanded the frontiers of subsurface microbiology and revealed novel pathways for energy acquisition.

Sources of organic matter

There are two main principles that bacteria employ when acquiring energy for fixing CO_2: phototrophy (photosynthesis) and lithotrophy (oxidation

and reduction of inorganic compounds) (Pace, 1997). Pace (1997) explains that lithotrophy extends broadly in the phylogenetic record of bacteria having a greater prevalence that phototrophy or organotrophy. This suggests that lithotrophy is far more widespread throughout the subterranean, and may extend kilometers into Earth's crust (Pace, 1997). There is a high probability that bacterial inhabiting anchialine caves utilize lithotrophy.

The benchmark study by Pohlman *et al*., (1997) on the organic cycling and ecology of anchialine caves using stable isotopes helped to clarify the trophic structure as well as the significance of chemosynthesis in anchialine environments. Although anchialine caves vary greatly in morphology, with Cherokee Road Extension Blue Hole being very different compared to that of Cenote Maya Blue (Pohlman *et al*., 1997), the potential sources of organic matter entering the systems should be similar.

Organic matter supporting anchialine ecosystems have long been attributed to being mainly detritus based (Dickson, 1975; Pohlman *et al*., 1997; 2000). Anchialine caves typically have some form of an entrance pool capable of supporting growth of various types of freshwater algae which has the potential to be transported into the passages below. Pohlman *et al*., (1997) identified such pools as a potentially significant source of organic matter. Cherokee Road Extension Blue Hole is a deep vertical collapse with an entrance pool directly above a vertically stratified water column. Currently there is little to no algal growth; however evidence of previous algal growth is observable. Large algal

stalactites (currently submerged) bend towards the entrance, formed when water levels were lower and drip water supported algal growth. Cherokee Road Extension Blue Hole is located in a pine forest with several tall grasses and rushes surrounding the entrance pool (Figure 2.2a), all of which could provide a source of organic matter to support the stygobitic fauna. The extent to which these sources contribute to the overall food web of Cherokee Road Extension Blue Hole is outside the scope of this project. However, several large tree branches are present at depth atop the talus mound illustrating the ease and presence of organic matter entering they system. With the prevalence of tropical storms in the Bahamas, deep vertical systems such as this should have a significant organic input consisting of sunken leaves and other detritus.

Transport of organic matter

Isotope analyses of soil particulate matter and cave sediments (Pohlman *et al*., 1997; 2000) were nearly identical, showing that as soil percolation enters the cave, this material sinks to the bottom and contributes to the cave benthic organic matter. It is important to understand that the overall quality of organic matter entering the cave system may be significantly reduced by retention of organics in the soil column, biotic molecular transformations, and/or by interstitial bacteria during its downward migration (Birdwell and Engel, 2010). While soil particulate matter was shown to be an importance source of organics, Poulson and White (1969) found that the mechanisms which transport substances into

subterranean systems are highly variable between caves and karstic regions. Meteoric water is undersaturated with CO_2 and $CaCO_3$ which initiates carbonate dissolution as it moves through the limestone. If meteoric waters were to percolate through every available joint and bedding plane present in a region then the lithology and geologic structure of the karst would look like “Swiss-cheese”, a pattern which is rarely observed (Poulson and White, 1969). Instead, soil organics as well as meteoric water enter through major drainage routes which continue to enlarge over time becoming large horizontal and vertical conduits becoming cave passages. Smaller joints and fractures may become plugged with sand, silts, and clays or remain small only seeing episodic water flow. Depending on the geologic history of the karstic region, the importance of soil organics may diminish over geologic time. This diminished importance is clearly the case with regards to organic matter entering by the intruding seawater. Pohlman *et al*., (1997; 2000) states how unlikely it is that any appreciable amount of usable organic matter is present in the intruding seawater after the long migration through the karstic aquifer.

Bahamian blue holes, much like the cenotes of the Yucatán, fluctuate tidally with the surrounding oceans. The carbonate island karst model (Figure 2.1; Mylroie and Mylroie, 2007) may provide a mechanism in which intruding seawater can play a significant role in providing organic matter to Bahamian caves over that present in Yucatán. Inland blue holes throughout the Bahamas discharge from diffusion zones found along the shelf margins (Cooper, 1959;

Gonzalez pers. obs., 2006). Compensatory flow of seawater into the cave systems from depth in the ocean is required to replace water lost through mixing zone discharge (Cooper, 1959; Whitaker and Smart, 1993). Active circulation of deep ocean water within the Bahamas carbonate platform, a process referred to as endo-upwelling (Whitaker and Smart, 1997), could transport nutrients from depths up to 1.8 km from the surrounding ocean and trenches. Such deep water circulation may explain the affinity of several bacterial clones from Cherokee Road Extension Blue Hole to bacterial clones from ocean environments below the photic zone.

Autochthonous inputs

The novel findings of Pohlman *et al*., (1997) in Yucatán show that several of the previously identified sources of organic matter may contribute to the overall source of carbon and nutrients in inland blue holes found throughout the Bahamas. There is however an overall heavy dependence on autochthonous inputs from chemoautotrophic bacteria from the interior of the cave that must not be overlooked. Anchialine caves are particularly well suited for chemoautotrophic processes with the multiple reactive mineral surfaces and the energetically rich waters (Pohlman *et al*., 2000; Birdwell and Engel, 2010).

Subterranean estuaries (defined in Chapter II) have unique biogeochemical conditions, primarily from lack of light. These subterranean estuaries are expected to be dissimilar from oceans, typical estuaries, and

freshwater systems at or near the surface (Birdwell and Engel, 2010). Understanding the source of dissolved organic matter in anchialine caves will delineate ongoing biogeochemical and ecological processes. In terrestrial estuary settings (non-subterranean), dissolved organic matter is derived from weathering processes and those from microorganisms. It is possible to differentiate between dissolved organic matter coming from the soil as opposed to that produced by chemoautotrophic bacteria within subterranean estuaries. Soil organic matter typically has high concentrations of organic acids such as humic acid and fulvic acid, which is generally lacking with chemoautotrophically produced organic matter in subterranean environments (Hugeuet *et al*., 2009; Birdwell and Engel, 2010).

Absorbance measurements of chromophoric dissolved organic matter provides a means to understand relationships and sources of dissolved organic carbon aquatic systems and is additional diagnostic tool in determining carbon sources. Initial case studies evaluated dissolved organic carbon in waters emerging from the Gironde Estuary (France). The Gironde Estuary has three salinity zones between upstream and downstream ranging in salinities from 0 ppt – 35 ppt. This particular estuary exhibited non-conservative mixing in which upstream organics did not undergo simple dilution as they moved downstream (Huguet *et al*, 2009). To help explain this phenomenon, chromophoric dissolved organic matter in the estuary was evaluated using the humification index (HIX) and the index of recent autochthonous contribution (BIX:

biological/autochthonous index). As the salinity gradient increased, HIX and BIX values shifted from having an important terrestrial contribution to that of a biological or aquatic bacterial origin (Huguet *et al*., 2009). The use of HIX and BIX indices are well suited to determine origin and aging of dissolved organic matter in aquatic environments.

Recently this method was employed to characterize dissolved organic matter in cave and spring waters, in addition to soil pore waters. While none of the cave systems investigated was an anchialine system, the findings show that previous theories on karstic hypogean systems are outdated and inaccurate. Samples collected from karstic and sulfidic karst springs around the world found their results to be consistent with those of Huguet *et al*., (2009) for autochthonous dissolved organic matter being microbially derived (Birdwell and Engel, 2010). All of the cave and spring samples showed low HIX values indicating that the chromophoric dissolved organic matter is of a recent biological source having significant contribution of carbon from *in situ* bacteria (Birdwell and Engel, 2010). In addition to investigating caves and spring waters, Birdwell and Engel (2010) also examined dissolved organic matter present in sediment porewaters. HIX and BIX values of sediment porewaters indicated that there was a combination of both allochthonous and autochthonous inputs. Using fluorescence as a means to characterize dissolved organic matter has strengthened and reaffirmed previous geochemical and molecular findings (Barns *et al*., 1994; Sarbu *et al*., 1996; Pohlman *et al*., 1997; 2000; Ley *et al*.,

2006; Macalady *et al*., 2006; Gonzalez *et al*., 2010) which shows *in situ* microbial activity contributing to the cave foodweb of both sulfidic and non sulfidic caves (Birdwell and Engel, 2010). The presence of dense microbial mats in Cherokee Road Extension Blue Hole suggests that aquatic microbes contribute to the overall organic matter as well as the overall carbon cycle. Molecular investigations of bacterial mats from Cherokee Road Extension Blue Hole (Figures 3.4-3.7) identified several types of bacteria which utilize varying modes of energy acquisition including anoxygenic photoautotrophy and lithoautotrophy, both capable of producing organic matter in situ. These findings strengthen the isotope findings by Pohlman *et al*., (1997) which indicate that both soil organics and chemosynthetic bacteria are the significant contributors to the carbon cycle in anchialine caves. Several students from the Marine Biospeleology Lab at Texas A&M University at Galveston plan to use radioactive and stable isotopes, as well as the methods used in Birdwell and Engel (2010), in order to form a more concise understanding of the source of carbon in anchialine caves. Such research, employing the use of chromophoric fluorescence, would be the first study of its kind from anchialine caves and should provide significant data on the nature of the organics supporting the trophic structure of anchialine caves.

Microbial mat community

The microbial mat present in Cherokee Road Extension Blue Hole is dark orange in color and exists in scattered patches on the walls below the halo/chemocline. The mat itself is several centimeters thick, spongy to the touch, and has small peak-like structures of unknown function protruding outward into the water column (Figure 3.9). Petroff *et al*., (2010) studying geometry of both modern and ancient conical stromatolites has found characteristic centimeter-scale conical protrusions similar to that found in the Cherokee Road Extension Blue Hole. Laboratory results indicate that these conical protrusions are derived under competition for limited nutrients and are capable of diffusing nutrients inwards. Since peak or conical-like structures form in nutrient limited pools with micro scale fluctuations, the structures observed from Cherokee Road Extension Blue Hole may be utilizing the same mechanisms. Upon dissection of the mat, there seems to be several layers each with a varying orange hue, potentially demarcating varying bacterial assemblages. Bacterial mats of similar color have been observed in other blue holes, as well as cenotes in Yucatán; however, none have been observed with the same denseness or topography (Gonzalez pers. obs., 2010).

Figure 3.9 Mat sample from Cherokee Road Extension Blue Hole. Note the peak-like structures protruding from the mat. Photo courtesy of Jenn Macalady, Penn State University.

The uniqueness of microbial mats, especially those from a depauperate environment raises questions to how these communities acquiring energy. According to Gause's Law, "…two species competing for the same resources cannot stably coexist if other ecological factors are constant…one of the two competitors will always overcome the other…." (Gause, 1934). Hutchinson (1961) deduced that vertical gradients and or some type of commensalism may overcome this ecological paradox. If environmental factors supporting the system are continually interacting with the community, then no single species is favored and an environmental equilibrium is never reached (Scheffer *et al*., 2003). Anchialine caves in general are deficient in nutrients and are characterized by environmental factors being relatively constant. Cherokee Road Extension Blue Hole in particular supports dense microbial assemblages

which appears to contradict Gause's Law. The studies of Barton and Jurado (2007) on microbial communities in terrestrial caves give insight on ways in which microbial communities in anchialine systems, especially that of Cherokee Road Extension Blue Hole can survive. Chemically complex nutrients in limited amounts are either entering Cherokee Road Extension Blue Hole from meteoric percolation, endo-upwelling, or from the limestone rock itself. Barton and Jurado (2007) explained that microbial mats are mutualistic associations of several species providing and utilizing nutrients to support growth which would be dampened if there was only selfish competition. 16s rRNA analysis of the mat in Cherokee Road Extension Blue Hole has yielded clones across a variety of bacterial groups, each with the own nutritional needs and methods of energy acquisition. Given the results present here and from other clone libraries constructed from neighboring Sawmill Sink, it is likely that a cooperative mutualistic community exists.

Phylogenetic profiles suggested by Barton and Jurado (2007) indicate that phenotypes within their cultures have the ability to fix nitrogen, metabolize complex aromatic compounds, and obtain minute amounts of trace nutrients from within the host environment. While these mechanisms (Barton and Jurado, 2007) pertain to terrestrial caves, they do illustrate alternative means for energy acquisition besides direct input. Furthermore, Pohlman *et al*., (1997) was able to isotopically identify several sources of organic matter within anchialine systems

and discovered the presence of a previously unknown chemosynthetic component.

Energy acquisition

The presence and exchange of atmospheric gasses is common throughout terrestrial systems as caves breathe when varying barometric pressure systems move over cave entrances (Barton and Jurado, 2007). With few exceptions, terrestrial caves have limitless supply of atmospheric gasses. Anchialine systems are only exposed to atmospheric gasses at the air-water interface of their entrance pool and in a more restricted way, at the surface of the water table. Cherokee Road Extension Blue Hole has a relatively large pool (11 m diameter and a circumference of 30.4 m) which may have localized wind induced mixing where oxygen is introduced into the upper freshwater layer, but input of CO_2 at the depth is limited to several biogeochemical processes which could introduce the gas at depth. Metazoan and microbial respiration during the aerobic breakdown of organic matter, as well as nitrate reduction during denitrification at the halo/chemocline, are capable of producing CO_2 into anchialine systems.

Soil derived aromatic compounds and particulate matter entering through meteoric percolation has been studied by Pohlman *et al*., (1997; 2000) and Birdwell and Engel (2010). Geologists studying flow paths of percolating groundwater in Carlsbad Cavern, New Mexico determined that nutrients may

take up to five decades to travel 300 m (Barton and Jurado, 2007). Nearly all anchialine systems in the Bahamas as well as in Yucatán are situated much shallower in the limestone terrain which would decrease the residency time of organics in the porespaces and allow nutrients to enter the system at an accelerated rate.

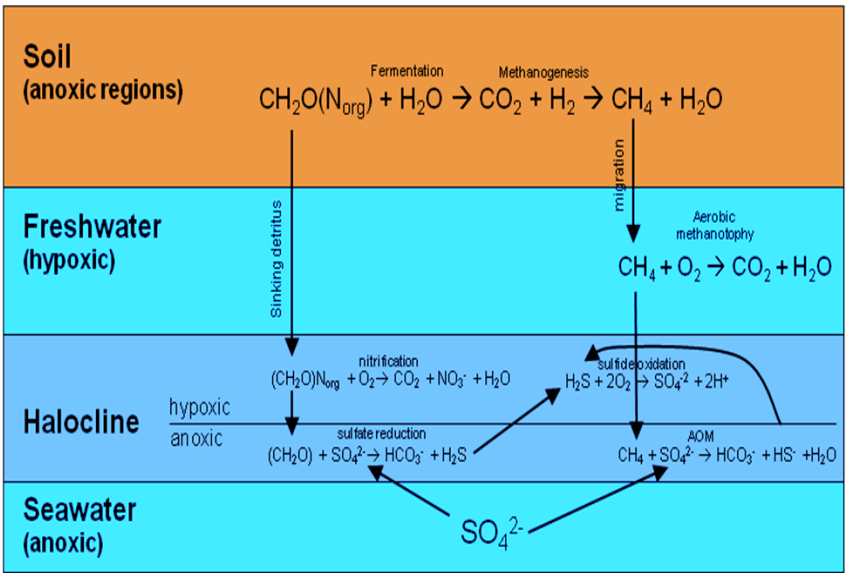

Figure 3.10 Diagrammatic overview of chemoautotrophy in an isolated/stressed anchialine cave. Designed by John Pohlman (2009).

As the water conditions change from oxic through suboxic to anoxic, the means of energy acquisition changes as oxygen is removed. The use of reduced metal ions (Mn(II) and Fe(II), for example) can provide energy for growth of lithoautotrophic bacteria. Many impurities are present in both the host limestone rock as well as in the secondary speleothem structures. Microbial species can and will breakdown the rock structures to access these minerals.

Mn, S, and Fe reduction are common biogeochemical processes within and below the halo/chemocline, but additional metals may provide a source of energy for microorganisms (Mills, 2002; Barton and, Jurado 2007). A theoretical model for chemoautotrophy in isolated or stressed anchialine systems was designed by John Pohlman (2009) (Figure 3.10). This figure ideally depicts to areas of a cave which are distant from cave entrances. Sinking detritus from the overlying soil is utilized in several different chemical reactions once it reaches the halo/chemocline, providing several potential sources of energy acquisition. Cherokee Road Extension Blue Hole may be considered an isolated or stressed system given the distance from the surrounding coast and the redox conditions present.

Reduction of metals in the environment is crucial to biogeochemical processes in the modern day biosphere. The reductions of several elements (Fe(III), Mn(IV), Cr(VI), U(VI)) are correlated with environmental cycling of carbon, oxygen, and sulfur (Ehrlich, 1978; Slobdokin 2005). In freshwater and marine systems, the reduction of Fe(III) and Mn(IV) by microorganisms allows for the oxidation of organic matter. Microbes have tremendous molecular and metabolic diversity, capable of exploiting any redox potential to support metabolism, often times affecting metal solubility within the environment (Southam, 2000). Extracellular polymeric substances (EPS) formed by the bacterial mat community are utilized in processes which dissolve and alter minerals in order to sequester nutrients locked within the rock (Walker and Pace,

2007). Cherokee Road Extension Blue Hole likely contains trace impurities of magnesium carbonate, silica, alumina, iron, sulfur, as well as many other minerals embedded in the limestone walls which microbes are capable of using as an energy source. Microbial corrosion of the limestone walls may provide a source of energy for several of the bacterial species present in the mat. The chief suspects in these microbial mediated corrosion processes are those associated with the sulfur cycle, which include the sulfur-oxidizers and sulfate-reducers (Hamilton, 1985; Iverson, 1987). Microbial induced corrosion (Figure 3.11) by sulfate reduces below the microbial mat (where oxygen is absent) would allow biodegradation of limestone impurities which could contribute resources for the rest of the consortium of mat bacteria.

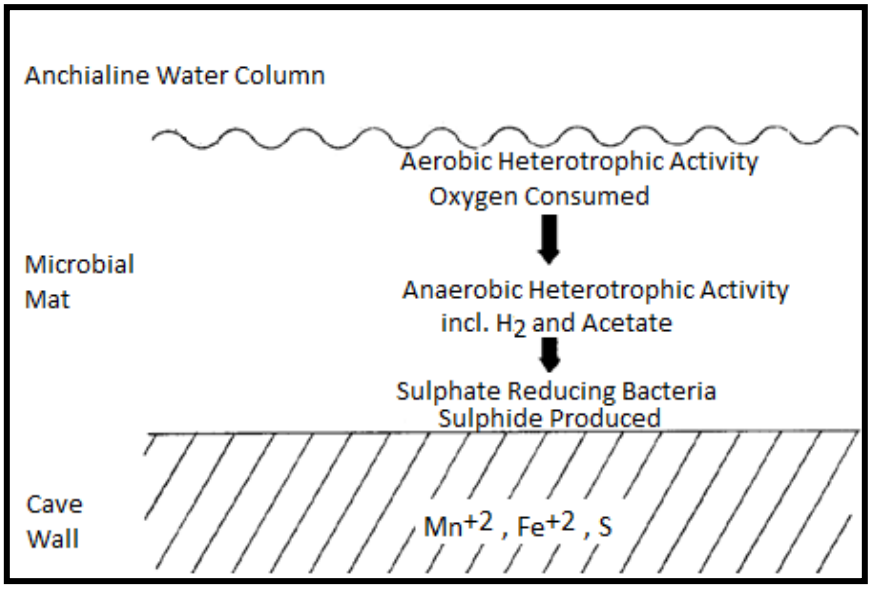

Figure 3.11 Microbially induced corrosion model redesigned for anchialine systems (Hamilton, 1985). Microorganisms depicted as the terminal link in the biodegradation of impurities found within limestone.

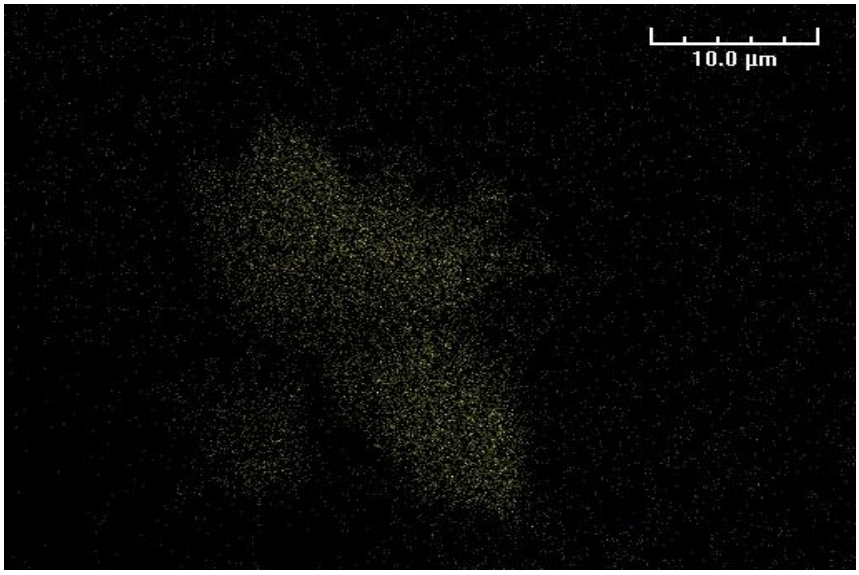

Figure 3.12 Energy-dispersive spectrometry (2700 x 30kv) of pyramidal crystal inclusions (see below Figure 3.13) from bacterial mat samples, identified as molybdenum. Microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

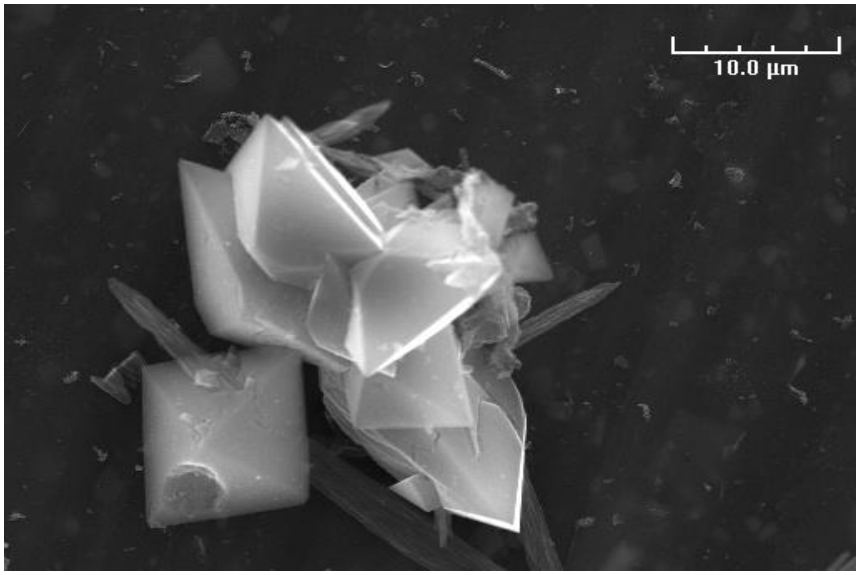

Figure 3.13 Scanning electron microscope image (2500 x 30kv) of pyramidal crystal inclusions from bacterial mat samples removed from Cherokee Road Extension Blue Hole. Scanning electron microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

Energy-dispersive spectrometry of bacterial mat samples from Cherokee Road Extension Blue Hole (Figure 3.12) showed that pyramidal crystal inclusions (Figure 3.13) within the mat had a molybdenum signature. Molybdenum is not usually found in large quantities nor commonly associated with bacterial oxidation. However, Mills (2002) suggested a chemical means by which microorganisms could potentially utilize what little molybdenum reserves are present:

$$MoS_2 + 5O_2 + 2H_2O \rightarrow MoO_4^{2-} + 2SO_4^{2-} + 4H^+$$

Molybdenum occurs naturally within the environment in several mineral forms and is a biologically important trace element (Southam, 2000). Molybdenite (MoS_2) is commonly oxidized aerobically into molybdate (Mo^{6+}) and sulfate which in turn can be reduced by microbes ($Mo^{6+} + e^- \rightarrow Mo^{5+}$) using sulfur as the electron donor (Southam, 2000; Slobodkin, 2005). The role in which molybdenum plays in the Cherokee Road Extension Blue Hole and the bacteria utilizing this element are still to be determined. This does however show that bacteria present in the Cherokee Road Extension Blue Hole is either utilizing impurities within the limestone or those that are being transported by the circulation of deep marine waters into the interior of the platform. Microbes capable of biomineralization (mineral formation) have been identified from several environments including fresh and saltwater, sediments, groundwater discharge zones, and hydrothermal systems (Southam, 2000).

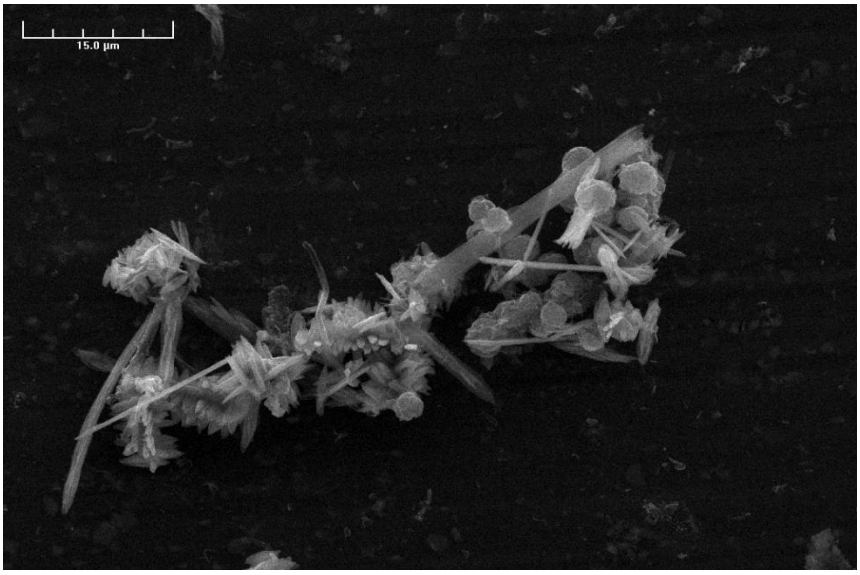

Figure 3.14 Scanning electron microscope image (1500 x 15kv) showing unidentified crystals and bacterial cells from Cherokee Road Extension Blue Hole. Scanning electron microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

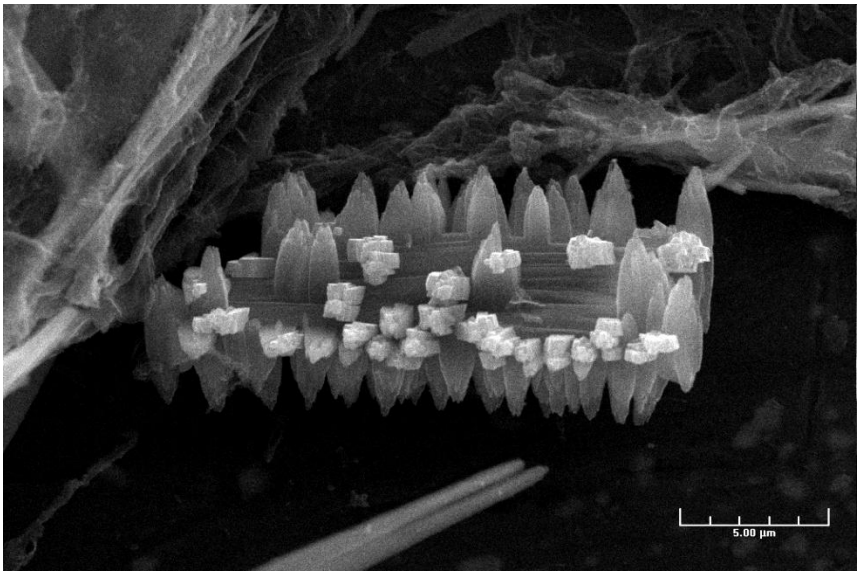

Figure 3.15 Scanning electron microscope image (1500 x 15kv) showing unidentified crystals and bacterial cells from Cherokee Road Extension Blue Hole. Scanning electron microscopy conducted through the Microscopy and Imaging Center at Texas A&M University, College Station, TX.

Scanning electron microscopy of mat samples from Cherokee Road Extension Blue Hole (Figures 3.14 and 3.15) found irregular crystals embedded within the mat matrix covered by bacterial cells. Preliminary analysis of the unidentified irregular crystals within the mat matrix using a raman microprobe has identified it as either jarosite ($KFe_3(SO_4)_2(OH)_6$) or natrojarosite ($NaFe_3(SO_4)_2(OH)_6$), which has been recently discovered on Mars. Both of these minerals are sulfate minerals which are present in areas where oxidation of iron sulfides occur. These two minerals are believed to be formed in wet oxidizing and acidic environments. These environments are possible to exist within the inner most mat structure closest to the rock surfaces where no oxygen is present. Biomineralization is well documented in several terrestrial caves as microbes obtain sources of energy within the structure of the host rock. Metal oxides accumulate as the rocks are broken down forming a powder known as moonmilk (Barton and Jurado, 2007). Significant numbers of iron and manganese oxidizing bacteria as well as species from the archaea group are present in moonmilk.

Biomineralization can also occur at depth deep within carbonate platforms forming sedimentary dolomite. Sedimentary dolomite is present in the geologic records beginning about 3.5 billion years ago. Some of the most studied dolomites have been from the Bahamas, with shallow dolomites of recent origin being discovered from several islands including Abaco (Sims, 1984). Dolomitization in carbonate platforms needs a constant supply of reactants

(primarily magnesium), and a geochemical environment suitable to allow dolomite to nucleate and grow (Simms, 1984; van Lith *et al*., 2000; Smith *et al*., 2002). Mass fluxes of magnesium and other minerals from the surrounding ocean by endo-upwelling provide the means for dolomitization to occur within the platforms of the Bahamas (Simms, 1984). Investigations of the geochemistry of cenotes in Yucatán have identified bacteria that play an essential role in sediment diagenesis (Smith *et al*., 2002). Sulfate reducing bacteria as well as thiosulfate oxidizing bacteria play an important role in reactions within the cenotes which drives early carbonate diagenetic processes such as limestone dissolution and dolomitization (Smith *et al*., 2002). Bacteria in anoxic conditions play a vital role in the evolution of carbonates throughout the geologic past (van Lith *et al*., 2000).

Bacterial diversity

The phylogenetic diversity of Cherokee Road Extension Blue Hole is of considerable interest and is a distinct microbial community. Interpreting the ecology of Cherokee Road Extension Blue Hole requires an in-depth understanding of bacterial mat communities as well as specific bacterial lineages, as several phylotypes showed notable sequence relationships to previous cultured and or environmental clones. Several clones also show no close relatives, representing potential new subdivisions.

The constructed dendrograms of the clones show a high relative abundance of *Chlorobium* and Candidate Division OP8 relatives (Figures 3.4 and 3.5). A high proportion of phylogenetically novel clones are present within the mat community in addition (Figure 3.6). Bacterial clones from a neighboring blue hole, Sawmill Sink, show affinities to several clones from Cherokee Road Extension Blue Hole. Although the biogeochemical nature and morphology of these two systems are characteristically different, the similarities between the two libraries suggest a heightened connectedness by which these bacteria are being transported. Phylogenetic analysis of aquatic microbial formations in the Nullarbor caves in Australia revealed several novel phlotypes which supports the unusual nature of these caves. The same unusual nature can be assigned to Cherokee Road Extension Blue Hole. The Nullarbor cave microbial community lacked or contained low abundances of several bacterial groups (*Actinobacteridae, Acidobacteria, α-Proteobacteria, Verrucomicrobia*) commonly represented in terrestrial soil bacterial communities (Holmes *et al*., 2001). Likewise, Cherokee Road Extension Blue Hole contains no clones with affinities to the previously mentioned bacterial groups. The clones which are phylogenetically novel sequences (Figure 3.6) may be similar to the *Verrucomicrobia* but show closer relations the bacterial group *Desulfacinum.* The few studies of cave-dwelling microorganisms in terrestrial caves showed that cave mud and clays being washed into caves contained well-known soil bacteria (Vandal, 1965; Barr, 1967). However, microbial mats from Cherokee

Road Extension Blue Hole and water samples from Sawmill Sink (Macalady *et al*. *in prep*) show that soil derived bacteria have a low abundance and or are absent altogether. Soil derived microbes may be of importance in terrestrial caves (Rusterholtz and Mallory, 1994; Engel and Northup, 2008) but lack such importance in anchialine caves. The bacterial clone library from Cherokee Road Extension Blue Hole identifies a bacterial community remarkably different from that of the terrestrial community. Pohlman *et al*., (1997) determined that soil derived organic matter percolating into the cave and chemoautotrophic bacteria were significant sources of nutrients. It is evident from his study and that of this one that those bacteria entering through percolation do not produce significant bacterial communities nor contribute to the community's consortium already present.

Holmes *et al*., (2001) suggested that such novelty amongst the subterranean clone libraries show parallels to those found by Hugenholtz *et al*., (1998) and may represent the rarely seen subsurface community. The Nullarbor caves have a high microbial biomass although little organic matter enters the system. This suggests that these Australian caves are supported by chemoautotrophy (Holmes *et al*., 2001). Several other aquatic caves, including anchialine caves, have reported similar findings each with a distinct mode of energy acquisition and varying levels of organic matter input (Barns *et al*., 1994; Sarbu *et al*., 1996; Pohlman *et al*., 1997; Hugenholtz *et al*., 1998; van Lith *et al*., 2000; Barton *et al*., 2004; Ley *et al*., 2006; Macalady *et al*., 2006; Seymour *et al*.,

2007; Birdwell and Engel, 2010). Results of 16s rRNA cloning suggest that the bacterial mat community present in Cherokee Road Extension Blue Hole utilizes anoxygenic photoautotrophy since the anoxygenic phototroph *Chlorobi* lineage is one of the most abundant clones in the 16s rRNA library. Additional evidence also suggests that chemolithotrophy plays a role amongst the other prevalent bacterial groups present in the mat.

On the basis of the phylogenetic survey, it is evident that the sulfur cycling plays an important role in the overall trophic structure in Cherokee Road Extension Blue Hole community. High levels of hydrogen sulfide are present (Figure 3.16) and increase with depth (Gonzalez *et al*., 2010). H_2S is a common byproduct of sulfate reducing bacteria. Other modes of energy acquisition should not be dismissed as there are several bacterial groups present within the mat community which may use alternative means and cycles. *Chlorobium* is prevalent in both Cherokee Road Extension Blue Hole and Sawmill Sink (Gonzalez *et al*., 2010*;* Macalady *et al*., in prep). *Chlorobium* has the ability to oxidize reduced sulfur compounds such as hydrogen sulfide during photosynthesis (Tabita and Hanson, 2004). Sulfate-reducing *Deltaproteobacteria* have also been found in the 16s rRNA libraries from both Sawmill Sink and Cherokee Road Extension suggesting that significant sulfur reduction occurs within their phototrophic microbial communities outside the photic zone of the caves.

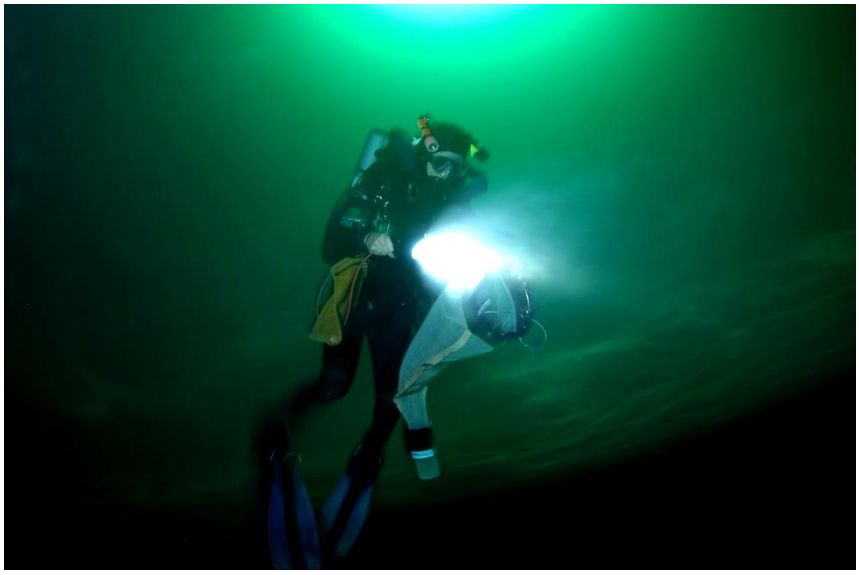

Figure 3.16 Rebreather diver Thomas Iliffe of Texas A&M University at Galveston tows a plankton net below the hydrogen sulfide layer where sunlight penetrates. Photo courtesy of Tamara Thomsen, Diversions Scuba, WI.

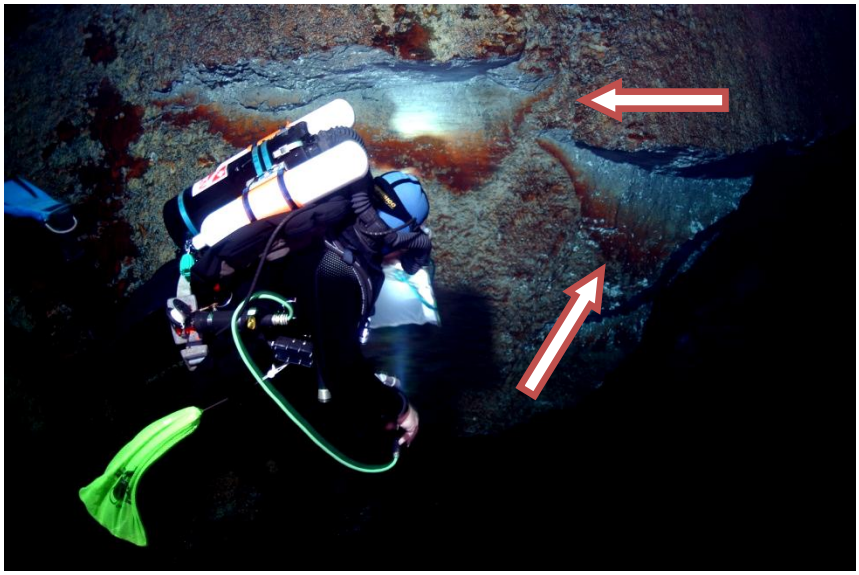

Figure 3.17 Rebreather diver Gregg Stanton (Wakulla Diving Center) towing a plankton net alongside the cave walls. Illumination of the cave walls shows selective mat growth (maroon arrows) in areas only with direct contact from sunlight above. Photo courtesy of Tamara Thomsen, Diversions Scuba, WI.

The growth of *Chlorobium* in Cherokee Road Extension Blue Hole is likely fueled by sulfide derived from reduction of seawater sulfate by other bacterial groups present in the blue hole. Considering the active deep water circulation within the carbonate platform, seawater sulfate reduction is continuous process. The microbial mat found in Cherokee Road Extension Blue Hole does not encircle the walls below the halocline; rather it is present only in certain areas where conditions allow the *Chlorobium* to thrive. Since *Chlorobi* are known for their ability to outcompete other phototrophs at low light intensities (Tabita and Hanson, 2004), they grow selectively on mat surfaces which are exposed to sunlight from above (Figure 3.17). Cave divers have observed that the bacterial mat does not grow in shaded areas under rock ledges or along the back of the talus mound.

In addition to the *Chlorobium*, the Candidate Division OP8 lineage showed an abundance of clones in the 16s rRNA library. The OP8 lineage was originally found in Obsidian Pool hot spring in Yellowstone National Park. Water in the pool is high in reduced iron and sulfur suggesting that the novel bacteria found in these hot springs are lithotrophic (Hugenholtz *et al*., 1998). Microbes which use lithotrophy significantly contribute to the overall chemistry of the biosphere (Hugenholtz *et al*., 1998). Lithotrophic bacteria acquire energy from inorganic chemicals such as hydrogen and reduced compounds and metals such as iron and sulfur and is phylogenetically more widely distributed than other metabolic metabolisms (Pace, 1997; Hugenholtz *et al*., 1998). With such an

abundance of clones of OP8 lineage, the presence of chemolithotrophy is most likely occurring below the surface of the mat at the interface between the cave walls and the mat.

An unknown component in the Cherokee Road Extension Blue Hole mat community is a filamentous organism which gives the mat its spongy nature. Holmes *et al*., (2001) found similar filaments imbedded within the bacterial mats which are reminiscent of *Thiothrix* or *Microthirx*. Although the identity of these filaments in Cherokee Road Extension Blue Hole is unknown, they are almost certainly biological and produced by microbes. Smears of the mat stained with DAPI (4, 6-diamidino-2-phenylindole), a fluorescent stain which binds to DNA, showed fluorescence within the unknown filaments. One of the clones represented in the library from the mat community was closely related to the *Chloroflexi* lineage. *Chloroflexi* are green non-sulfur bacteria which form dense filamentous strands. These bacteria are typically facultative aerobes, but, considering the dominance in the mat by *Chlorobium*, they may be in a non active state. Regardless, it is evident that these filaments do not amplify effectively with the primer set used and or there are additional substances which are inhibiting the amplification. Considering the density of the mat and the filaments, DNA yields were relatively low.

It is clearly evident that there are many unanswered questions with regards to the bacterial diversity of anchialine systems and how the bacterial composition regulates biogeochemical processes and contributes to the overall

trophic structure. The community structure of the Cherokee Road Extension Blue Hole mat is unique amongst known microbial habitats. It is particularly interesting to find that even though there is high organic matter input and a direct surface connection, the bacterial composition in Cherokee Road Extension Blue Hole lacks terrestrial constituents. Given the concentration of hydrogen sulfide in the water, it is feasible that the microbial mats in Cherokee Road Extension Blue Hole represent a novel anoxygenic photoautotrophic bacterial community dominated by *Chlorobium* lineage in the upper portion of the mat with a chemolithotrophic bacterial community dominated by OP8 bacterial lineage at the base of the mat.

CHAPTER IV

FINAL SUMMARY AND CONCLUSIONS ON THE BACTERIAL DIVERSITY OF CHEROKEE ROAD EXTENSION BLUE HOLE

Study Rationale

The main rationale for this study was to investigate the unique assemblage of bacterial mats present in Cherokee Road Extension Blue Hole on Abaco Island, Bahamas. Work set forth examined the following hypotheses: 1) anchialine blue holes have monospecific bacterial assemblages; 2) bacteria found in anchialine caves consist of previously identified taxa that are commonly associated with terrestrial soil habitats; and 3) bacteria found in caves are cave specific. In addition to the three hypotheses tested, hydrological data collected during the expedition suggested a need for a reexamination of the dynamic balance of fresh and saltwater present in anchialine inland blue holes. Genetic analysis of the bacterial mats used to test the hypotheses represents one of the few studies utilizing 16s rRNA clone libraries to look at the bacterial diversity in anchialine blue holes of the Bahamas.

Findings

Hydrological parameters

Chapter II examines the physico-chemical profiles of the water column in Cherokee Road Extension Blue Hole. Physical and chemical water parameters

of Cherokee Road Extension Blue Hole and those neighboring were found to have the same vertically stratified water columns similar to anchialine caves throughout the world. It is within these water columns that sets each anchialine system apart from the next. Within Cherokee Road Extension Blue Hole, small scale hydrologic variations occur with measured parameters such as temperature, DO, and ORP which act in response to the translucent hydrogen sulfide layer. This layer of hydrogen sulfide has a profound effect on the cave morphology as well as the anchialine ecosystem, governing not only the microbial mat diversity below the halocline but the stygobitic community throughout the blue hole.

The pronounced stratification of the hydrologic parameters measured in Cherokee Road Extension Blue Hole is only a glance at the true complexity of the vertical stratification within Cherokee Road Extension Blue Hole and anchialine systems altogether.

Cherokee Road Extension Blue Hole was one of six holes sampled during the March 2006 expedition. Three of the six blue holes (including Cherokee Road Extension Blue Hole) had little to know horizontal passageway, yet still showed responses to daily tidal oscillations and exhibited the same vertically stratified water column. Cherokee Road Extension Blue Hole has several distinct water demarcations within the water column which include a thermocline beginning at approximately12.5 m (as seen in Figure 2.3), a halocline at 13.7 m (as seen in Figure 2.4) which overlays a hydrogen sulfide layer at ~25 m. As

you descend through the water column, hydrological parameters such as dissolved oxygen (DO), pH, and ORP are also affected by water column complexity. DO levels vary greatly between entrance pool (oxic) and maximum measured depth (dysoxic) (as seen in Figure 2.5), pH values show two major shifts lowering the pH values in response to the halocline and the hydrogen sulfide layer (as seen in Figure 2.6). In addition, ORP values are distinctly bimodal showing higher oxidative values in the shallower freshwater layer, while negative reducing conditions were present at the deeper depths (as seen in Figure 2.7).

To help interpret the results, plots of the constituent concentration versus salinity (conservative mixing diagrams) were developed to further elucidate the ongoing processes within the water column at Cherokee Road Extension Blue Hole. It is evident from the YSI sonde data that water within Cherokee Road Extension has warmed since entering the groundwater (as seen in Figure 2.8a). If we look at the individual water layers (as seen in Figure 2.8b-d) we see that the freshwater and saltwater layers are cooler than the water across the halocline. This overall warming effect can be attributed to microbial mediated processes occurring at the transition zones while the cooler deeper water is in response to circulation of deep ocean water within the carbonate platform. Using the same technique to examine hydrogen ion concentrations (as seen in Figure 2.9b-d), we see that there is a source for hydrogen ions across the freshwater and halocline. This may be in response to the production of H_2S

from the breakdown of organic matter. Anchialine caves are typically associated with decreased levels of dissolved oxygen at depth due to absence of photosynthetic oxygen production and reduced vertical mixing which restricts exchange of oxygen at surface waters. The mixing diagrams for DO (as seen in Figure 2.10b-d) show that there is an oxygen sink within the fresh and saltwater layers, but across the halocline, DO acts conservatively. The conservative mixing of DO across the halocline may be due to lateral flow of water within the carbonate platform as it exits through the zone of diffusion.

A common misunderstanding assumes salt water in coastal aquifers is static beneath carbonate platforms (Whitaker and Smart 1990). In the presence of a zone of diffusion at the halocline, salt water is not static but in a perpetual cycle from the sea to the zone of diffusion and back. If a static state were to occur in the carbonate banks of the Bahamas, geothermal heat flux similar to that of the adjacent Florida peninsula (1.0°C/40 m) should exist and a general warming trend would be detected at depth in all inland blue holes (Whitaker and Smart 1993). Our observations (as seen in Figure 2.15) as well as those by Whitaker and Smart (1990, 1993) suggest a cooling effect rather than a warming effect due to deep water circulation and or endo-upwelling. Blue holes which show a warming effect are often associated with other processes, such as reducing environments and abundant bacterial populations, which would allow heat generation to occur.

Examining sonde data (unpublished data) gathered from other blue holes investigated in the surrounding areas show similar hydrologic results which point to a non-static saltwater state. For example, dissolved oxygen levels in Sawmill Sink Blue Hole first drop to zero within a dense opaque hydrogen sulfide layer at the halocline, then recover in the fully marine waters beneath it. No circulation of surface water penetrates the H_2S layer in Sawmill Sink. Dan's Cave (as seen in Figure 2.12b-d), is a blue hole with predominantly horizontal passageways which has a clearly defined warming of waters present at the halocline, yet cooler temperatures in both the freshwater and saltwater layers. There is no bacterial mat within Dan's cave; however water temperatures at depth are cooler than the annual air temperature of the region. Temperatures at depth should mirror the average annual air temperatures of the region as is the case for terrestrial dry caves. The temperature sink in the saltwater layer in Dan's Cave and the recovery of dissolved oxygen in Sawmill Sink further contributes to the idea behind deepwater circulation within the carbonate platforms of the Bahamas.

Analysis of the hydrological data reveals that further investigation and long term monitoring is needed to precisely identify the mechanisms driving the physico-chemical profiles associated with Cherokee Road Extension Blue Hole. Measured water quality parameters need to be coupled sampling of chlorophyll, hydrogen sulfide, acetate, sulfate, and nitrate.

Whitaker and Smart (1997) have proposed circulation of cold ocean waters deep within carbonate platforms. The data collected during the Abaco expedition indicates that similar phenomena may occur within the Little Bahama Bank platform. Additional physico-chemical measurements of waters discharging from oceanic blue holes, cracks, and fissures along the platform margins are needed for conclusive evidence of this circulation mechanism.

Bacterial mat analysis

The main scope of this project was to elucidate the biogeochemical processes occurring in anchialine blue holes by assessing the genetic diversity of a microbial mat found in Cherokee Road Extension Blue Hole, Abaco Island, Bahamas (see Chapter III). Inland blue holes of the Bahamas typically show redox stratification, many of which are associated with sulfidic layers; yet the biogeochemical cycling is complex and poorly understood. There are only a few previous works (i.e., Dickson, 1975; Humphreys, 1999; Schwabe and Herbert, 2004; Herbert *et al*., 2005) on the microbiology of anchialine systems (cenotes and blue holes) which were based solely on microscopy and culturing. Both of these methods introduce biases because culturing practices are often impractical since replication of the cave environment is nearly impossible and ~90-99% of microbes are unculturable (Engel and Northup, 2008). This investigation is one of a few (i.e., Holmes *et al*., 2001; Gonzalez *et al*., 2010; Macalady *et al*., in prep) which utilize 16s rRNA clone libraries to identify

microbial mat diversity and its relationship to the biogeochemical processes ongoing in anchialine blue holes.

Bacterial mat samples (collected from 40.8 m) were obtained below the halocline in Cherokee Road Extension Blue Hole. Fragments (approximately 3 mm^3 in volume) of the bacterial mat sample collected from 40.8 m water depth was prepared and used for nucleic acid extraction and amplification using a bead beating protocol (Appendix II) to extract the environmental DNA and prepare it for downstream applications which included cloning and DNA sequencing.

A total of 65 non-chimeric 16s rRNA sequences were aligned and used for construction of dendrograms. Appendix IV lists the entire diversity of sequenced cloned as well as percent similarity to both cultured and uncultured bacteria previously reported in GenBank (http://www.ncbi.nlm.nih.gov). The community structure of the phylotypes present in Cherokee Road Extension Blue Hole is listed in Figures 3.4 - 3.7. A distinctive feature of this mat is the distribution of phylotypes across major subdivisions in the bacterial domain. These blue hole phylotypes fall into divergent branches which include previously described groups well defined by culture and or culture independent means. These branches include *Chlorobium* and Candidate Division OP8. Additional clones were grouped amongst a long lineage of bacteria, but did not align to any other known group and thus may represent novel subdivisions with relative similarity to the bacterial groups Defferibacter, Fibrobacteres, Verucomicrobium

and Desulfacinum (Figure 3.6), all which are capable of reducing sulfur, sulfate, and nitrate.

Results of 16s rRNA cloning suggest that the bacterial mat community present in Cherokee Road Extension Blue Hole utilizes two routes of energy acquisition; 1) anoxygenic photoautotrophy and 2) chemolithotrophy. Anoxygenic photoautotrophy is occurring throughout the upper portions of the mat by the *Chlorobi* lineage which is taking advantage of the light entering through the cave pool while the OP8 lineage is occupying the areas between the mat and the cave walls where reduced metals are being utilized.

Tested Hypotheses

Monospecific bacterial assemblages

During the initial exploratory cave dives in Cherokee Road Extension Blue Hole, the bacterial mat was observed to be several centimeters thick, spongy to the touch, and had small peak-like structures of unknown function protruding outward into the water column (Figure 3.9). The mat is dark orange in color and only exists in areas which are illuminated by the sun, appearing to have selective mat growth. Bacterial mats of similar color have occasionally been observed in other blue holes throughout the Bahamas Archipelago, as well as in a few number of cenotes (Cenote Crustacea, Cenote Angelita) in the Yucatán Peninsula. It is still unknown what drives perpetuates that production of mats in anchialine caves.

Previous studies on the biogeochemical ecology of underwater limestone caves have found visible bacterial colonies which belong to a single species, usually that of *Thiothrix* spp. or *Beggiatoa* spp., a commonly occurring bacteria in Floridian freshwater springs (Brigmon *et al*., 1994). Since anchialine caves, like Florida springs, typically have low organic nutrient levels, common in environments lacking photosynthesis, there was a possibility that the mat within Cherokee Road Extension Blue Hole would be dominated by a single bacterial species similar to that of *Thiothrix* spp., or *Beggiatoa* spp.

Dissection of the mat collected on subsequent trips revealed it is made up of several layers each with varying orange hues, potentially demarcating various bacterial assemblages. While clone libraries were not constructed using the varying color layers, cloning experiments did reveal a diverse assemblage of bacteria across several major bacterial lineages, each with their own nutritional needs and methods of energy acquisition. These results show that the bacterial mat present in Cherokee Road Extension Blue Hole is not monospecific and is likely a cooperative mutualistic community.

Rarefaction curves and coverage calculations of the16s rRNA clones indicates that the original clone library was too small to obtain a complete picture of the true bacterial diversity. Results indicate that only a fraction of the total bacterial population of the mat was observed. This indicates that the true nature of the bacterial mat present in Cherokee Road Extension Blue Hole is

considered to be complex with high diversity of taxa and not that of a monospecific colony.

Bacteria common to terrestrial soil habitats

The subterranean environment has long been viewed as an extension of the surface world. Initial investigations showed that soil heterotrophs, as well as chemoorganotrophs, and fecal coliforms were translocated into non-anchialine caves and made up the bacterial community (Rusterholtz and Mallory, 1994; Engel and Northup, 2008). Caves in general have been viewed to have a heavy dependence on soil organics. These soil organics were shown to play a significant role in the food web in cenotes of the Yucatán Peninsula (Pohlman *et al*., 1997; 2000). With such a heavy dependence in the subterranean on terrestrial soil inputs, one would expect to find a significant presence of common soil bacteria (*Actinobacteridae, Acidobacteria, α-Proteobacteria, Verrucomicrobia*) within the bacterial community of caves. Holmes *et al*., (2001) used techniques similar to this study to investigate the phylogenetics of unusual aquatic microbial mantles in the Nullarbor Caves in Australia. Their results found no such presence of the before mentioned bacterial groups common to soil communities. They did however find a high standing microbial biomass comprised of 16 bacterial lineages including the α,γ,δ-Proteobacteria, Nitrospira, and Actinobacteridae. DNA sequences from the Cherokee Road Extension Blue Hole clones showed that the microbial community either lacked or contained

extremely low abundances of commonly associated terrestrial soil bacterial groups. Furthermore, of the 65 clones used in the final analysis, 22 lack a close affinity to any major bacterial lineage and may represent a potentially novel lineage. These 22 clones (Figure 3.6) may be similar to the *Verrucomicrobia* (a common terrestrial bacterial group); however, they show a closer relationship the bacterial group *Desulfacinum*, uncommon to terrestrial soils.

Additional molecular evidence from anchialine blue holes on Abaco Island, Bahamas, suggests that common terrestrial soil bacteria play a very little role in anchialine blue holes. Sawmill Sink, a neighboring inland blue hole located 20 km to the southwest of Cherokee Road Extension, not only has an extremely dense H_2S layer, but also contains oxygenated waters below the H_2S that support a diverse stygobitic invertebrate assemblage. The H_2S layer appears as a purplish-brown to reddish-brown planktonic assemblage or bacterial plate that is situated atop the halocline. Similar microbial investigations to the ones from Cherokee Road Extension Blue Hole have been performed on filtered water samples from Sawmill Sink (Macalady *et al*., in prep.) and show that common soil derived bacteria have an extremely low abundances with many of the common groups absent altogether. Interestingly enough, water from the halocline region in Sawmill Sink contains many of the same major bacterial groups that are prevalent in the mat from Cherokee Road Extension Blue Hole. Soil derived microbes may be of importance in terrestrial caves (Rusterholtz and

Mallory, 1994; Engel and Northup, 2008) but thus far lack such significance in anchialine inland blue holes.

Endemism in cave microbes

Stygobitic fauna found in anchialine caves is highly diverse and usually endemic to a region and even a specific cave (Iliffe and Bishop, 2007). It has been shown that chemosynthetic bacteria contribute a significant portion to the trophic level as well as to the overall anchialine environment (Pohlman *et al*., 1997), the question remains however if the bacteria which contribute to the overall function of the anchialine ecosystem show the same endemic nature as the fauna.

The bacterial clones from Cherokee Road Extension Blue Hole represent novel strains for known bacterial lineages. These new strains do however represent an extension to their known habitats being the first to identify them in an anchialine cave environment. They two major bacterial lineages observed in Cherokee Road Extension Blue Hole (*Chlorobium* and Candidate Division OP8) are of importance for studies of the early Earth and suggest that anchialine caves may serve as potential models to the past Proterozoic (geological eon representing a periods before the first abundant complex life form) oceans whereby anoxygenic photosynthesis modulated Proterozoic oxygen (See concluding remarks).

Bacterial clones from a nearby blue hole, Sawmill Sink (Macalady *et al*., in prep.), were compared to those from Cherokee Road Extension Blue Hole (Figure 3.7) with six clones found to be similar, while several others show close relatedness. This initially disproves the idea that each cave has an endemic bacterial presence; additional sampling will need to be done to verify confirm an endemic bacterial presence. It does however raise additional questions regarding how inland blue holes over 20 km apart have such similar microbiota. Perhaps both Sawmill Sink and Cherokee Road Extension Blue Hole are one extensive and integrated hydrological system. Various theories which could explain such similarities need to be investigated. These theories include deep water circulation or endo-upwelling deep within the platform which transport the microbiota upwards from great depths; transference of the microbiota by lateral transport of water within the platform; independent colonization of inland blue holes as conditions arose; ancient origins under conditions potentially similar to those of early Earth; or all blue holes on Abaco Island or even in the Bahamas have similar microbiota.

The microbiota of Cherokee Road Extension has clearly not been surveyed comprehensively by culture-independent means which would be needed to fully understand the trophic dynamics and biogeochemical processes ongoing within anchialine ecosystems as a whole. Further molecular investigations throughout the Bahamian blue holes should determine if the

bacteria present on Abaco Island are localized or scattered throughout the archipelago.

Implications

Our results substantiate previous findings that anchialine caves and inland blue holes have a widely varying and complex biogeochemistry. This variation and complexity makes anchialine caves and those found on Abaco Island in particular, valuable resources as natural laboratories which can reveal constraints on the biogeochemistry of stratified Proterozoic oceans prevalent in early Earth history (Reinhard *et al*., 2009).

New findings reveal that persistent oxygenation of oceanic water masses during the Proterozoic occurred much later (580-550 Ma) than that of the atmosphere and surface ocean (2.4 Ma) (Scott *et al.,* 2008; Johnston *et al.,* 2009). Oceanic surface waters present in Earth's middle ages overlaid an oxygen minimum zone where euxinia (i.e., paired anoxic and sulfidic conditions) persisted below (Johnston *et al.,* 2009), very similar to those of modern day anchialine cave systems. There are only a few extant euxinic basins , with the Black Sea and Cariaco Basin being the largest. Continual euxinia is viewed as an extreme endmember of sulfidic conditions (Scott *et al.,* 2008). It is important to note that conditions found in present day anchialine caves scattered throughout the Bahamas and Yucatán provide accessible sites for studying this phenomenon.

Throughout much of the Mid-Proterozoic, anoxygenic photoautotrophs were responsible for the primary production and biogeochemical cycling (Johnston *et al*., 2009). Using sulfide as an electron donor sustained euxinia and generated organic matter. Our cloning results suggest that the same processes occur within Cherokee Road Extension Blue Hole and Sawmill Sink, further illustrating how these systems can be utilized as modern analogs of microbial ecosystems occurring before and during the Proterozoic production of oxygen. The discovery of microbial mats dominated by anoxygenic phototrophs such as *Chlorobi* within two anchialine caves on one island provides unequivocal evidence towards anchialine caves being true analogs to Proterozoic oceans. As we continue to investigate anchialine caves, one wonders what other biogeochemical processes might be similar to those of Proterozoic oceans.

Identification of crystal inclusions within the microbial mat found below the halocline in Cherokee Road Extension Blue Hole indicates that process are occurring within the mat to sequester reduced elements from the host rock, the intruding seawater or a combination of both. Energy dispersive spectrometry identified the pyramidal crystals as molybdenum. Oxidative weathering of continental crusts releases molybdenum which is transported via rivers into surrounding oceans (Arnold *et al.,* 2004; Scott *et al.,* 2008). The Bahamas archipelago has no significant rivers and likewise lacks continental crusts which could introduce molybdenum into the anchialine systems. These findings indicate the molybdenum is either an impurity within the limestone or is being

transported upwards into the blue holes through deep water circulation within the carbonate platforms. Within modern oceans, molybdenum is one of the most abundant transition metals (0.01 ppm), however under settings of euxinia (where H_2S >100 μM), molybdenum is removed from solution (Arnold *et al.,* 2004). Cherokee Road Extension Blue Hole has H_2S levels ~400 μM at depth (Gonzalez *et al.,* 2010) which would explain how these crystals were come out of solution. Arnold *et al.,* (2004) has found that the overall molybdenum budget is redox sensitive. Given these findings and those from cultured experiments, settling of molybdenum into sulfidic environments most definitely could have restricted the evolutionary path of eukaryotes Scott *et al.,* 2008). Culturing experiments show that growth rates of cyanobacteria are affected by molybdenum limitation by directly affecting nitrogen fixation. Given the availability of redox sensitive bioessential metals (Fe and Mo) in Cherokee Road Extension Blue Hole, it leads us to questions the common assumption that biological N_2 fixation precludes N limitation (Anbar and Knoll, 2002). Cherokee Road Extension Blue Hole has a bioinorganic bridge whereby the understudied cycling of molybdenum is present amongst dense microbial assemblages of anoxygenic phototrophs.

Subsequent Work

In December 2008 and in July and December 2009, research divers on a National Geographic supported project and microbiologists from Pennsylvania

State University revisited Abaco Island to investigate if blue holes could be used as analogs for Proterozoic oceans. Sawmill Sink and Cherokee Road Extension Blue Hole, both having conspicuous phototrophic communities and contrasting geochemistry, were used for further investigation of these early Earth models. Microbial plate and mat samples were collected from both systems and used to develop 16S rRNA clone libraries.

Sawmill Sink

Sawmill Sink has a dense purplish-brown to reddish-brown microbial plate which is present throughout the year atop the halocline. Microbial plates present in a stratified lake on Andros Island have the same coloration and are dominated by members of the *Gammaproteobacteria* (Schwabe and Herbert, 2004). Results from culture-independent analysis showed the great majority of the clones in Sawmill Sink are affiliated with *Chlorobi* (Figure 4.1), an obligate photoautotroph lineage of bacteria. *Chlorobi* also make up >50% of 16S rRNA clones obtained in clone libraries from filters collected in other seasons and from deeper depths within the halocline (Macalady *et al*., in prep.).

Cherokee Road Extension Blue Hole

In January of 2010, I traveled to Pennsylvania State University to work up samples collected from Cherokee Road Extension Blue Hole which had been collected during the July and December 2009 expeditions. Using the same

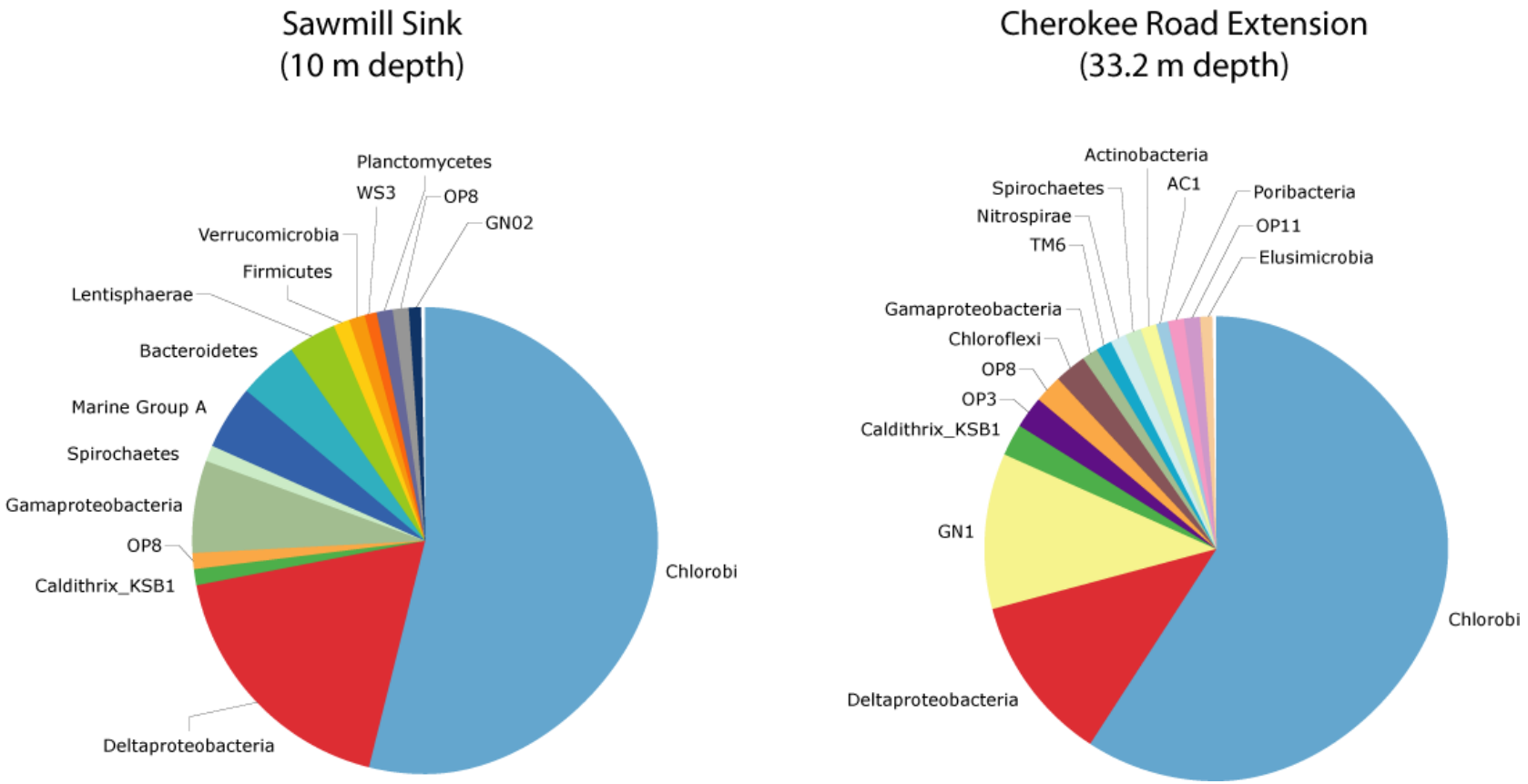

Figure 4.1 Population structures of Sawmill Sink and Cherokee Road Extension Blue Hole microbial communities based on bacterial 16S rRNA cloning.

techniques used in the Sawmill study, two additional bacterial clone libraries as well as one archeal library were constructed from bacterial mats at 24.4 m and 33.2 m depths in Cherokee Road Extension Blue Hole. Members of the anoxygenic phototroph *Chlorobi* lineage (Figure 4.1) are the most abundant clones in 16S rRNA libraries constructed from samples collected across the depth range where the wall mat occurs. The combined results from the varying clone libraries will enable us to observe spatial differences in the microbiota.

Preliminary results from pigment analysis indicate that there are no photosynthetic chlorophyll pigments within the mat but several bacteriochlorphylls. FISH (fluorescence *in situ* hybridization) was also performed on bacterial mat samples from Cherokee Road Extension Blue Hole and was found to not be affective as the sample auto fluoresces naturally.

The geochemical profiles of Cherokee Road Extension Blue Hole and Sawmill Sink are drastically different; however, sulfate-reducing *Deltaproteobacteria* are abundant in both 16S rRNA libraries (Figure 4.1). This evidence points towards significant sulfur reduction within the phototrophic microbial communities as well as within the deeper water outside the photic zone of these inland anchialine blue holes (Gonzalez *et al*., 2010).

Concluding Remarks

Throughout this study, a number of additional questions were raised while examining the results which indicate further analysis is needed to fully grasp the

microbial ecology and biogeochemistry within the inland anchialine blue holes of the Bahamas. Long term deployment of sonde's and current meters in addition to water sampling from boreholes, inland and oceanic blue holes, cracks and fissures around the margins and deep seas surrounding the islands need to be conducted to determine the existence of deepwater circulation within the various platforms of the Bahamas Archipelago. Reassessment of the dynamic balance of fresh and saltwater in carbonate platforms can be used to better understand the varying biogeochemical nature of anchialine inland blue holes.

The field of microbial ecology in caves is relatively undeveloped compared to that of macro ecology and even more so in the anchialine environment. Many fundamental ecological questions regarding anchialine systems remain unanswered in regards to food web dynamics, bacterial mediated processes, and availability of both organic and inorganic sources of nutrients. The primary purpose of microbial ecology is to understand the relationships between microbes and the environment (Walker and Pace, 2007). Many aspects of the microbial ecology and biogeochemistry of anchialine caves remain to be investigated. Our knowledge of the subterranean biosphere is continuously expanding yet many unanswered questions linger in regards to energy acquisition in deep caves and rocks, and what are the genetic and physiological differences between surface and subsurface microbes, as well as how metabolically active are the deep cave microbiota. Given the number of

anchialine caves scattered throughout the world, there is an ever abundant amount of fertile ground for amazing discoveries.

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APPENDIX I

YSI SONDE SPECIFICATIONS

YSI 600XL & 600XLM Sensor Specifications			
	Range	Resolution	Accuracy
Dissolved Oxygen % Saturation 6562 Rapid Pulse™ Sensor*	0 to 500 %	0.1%	0 to 200%: ±2% of reading or 2% air saturation, whichever is greater; 200 to 500%: ±6% of reading
Dissolved Oxygen mg/L 6562 Rapid Pulse™ Sensor*	0 to 50 mg/L	0.01 mg/L	0 to 20 mg/L: ±0.2 mg/L or 2% of reading, whichever is greater; 20 to 50 mg/L: ±6% of reading
Conductivity 6560 Sensor*	0 to 100 mS/cm	0.001 to 0.1 mS/cm (range dependant)	±0.5% or reading + 0.001 mS/cm
Salinity	0 to 70 ppt	0.01 ppt	±1% of reading or 0.1 ppt, whichever is greater
Temperature 6560 Sensor*	-5 to +50°C	0.01°C	±0.15°C
pH 6561 Sensor*	0 to 14 units	0.01 unit	±0.2 unit
ORP	-999 to +999 mV	0.1 mV	±20 mV
Depth & Level			
Medium	0 to 200 ft, 61 m	0.001 ft, 0.001 m	±0.4 ft, ±0.12 m
Shallow	0 to 30 ft, 9.1 m	0.001 ft, 0.001 m	±0.06 ft, ±0.02 m
Vented Level	0 to 30 ft, 9.1 m	0.001 ft, 0.001 m	±0.01 ft, ±0.003 m

*Report outputs of specific conductance (conductivity corrected to 25° C), resistivity, and total dissolved solids are also provided. These values are automatically calculated from conductivity according to algorithms found in *Standard Methods for the Examination of Water and Wastewater* (ed 1989).

APPENDIX II

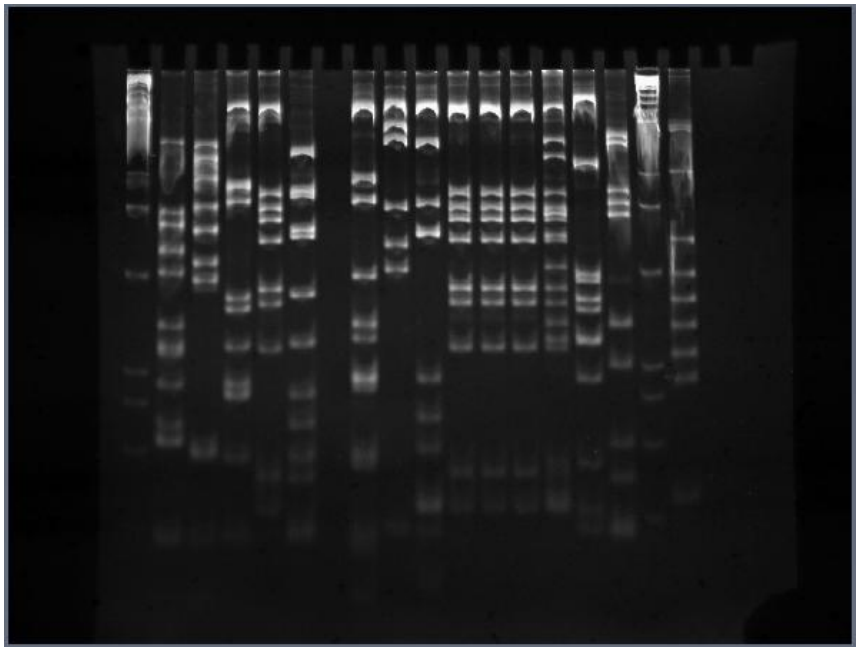
EXTRACTION PROTOCOL

- 500 ml 2X Buffer A (NaCl 200 mM (1.16g/100 ml), Tris 200 mM (2.42 g/100 ml) EDTA 20 mM (from conc. Stock))
- 200 ml 20% SDS
- Zirconium Beads
- 30 min at 37 °C
- 500 ml 1:1 Phenol:Chloroform
- Bead Beat 2 min
- Spin, 13,000, 4 min
- Aqueous Layer (Top Layer) out to new Eppendorf Tube
- 500 ml 1:1 Phenol:Chloroform
- Mix by Inversion
- Spin, 13,000, 4 min
- Aqueous Layer out
- Precipitate with 600 ml cold Isopropanol & 60 ml 3M Na-Acetate
- Ice, 20 min
- Spin, 20 min
- Decant
- 500 ml cold 100% Ethanol
- Decant
- Air dry, inverted, 20-30 min
- Suspend in 20 ml TE

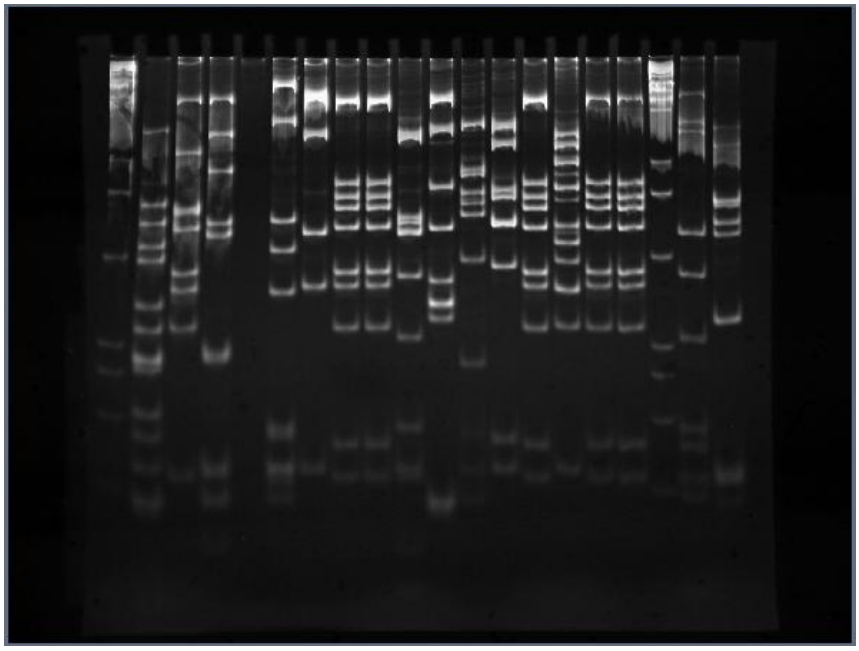
APPENDIX III

MICROBIAL MAT ARDRA PATTERNS, CHEROKEE ROAD EXTENSION

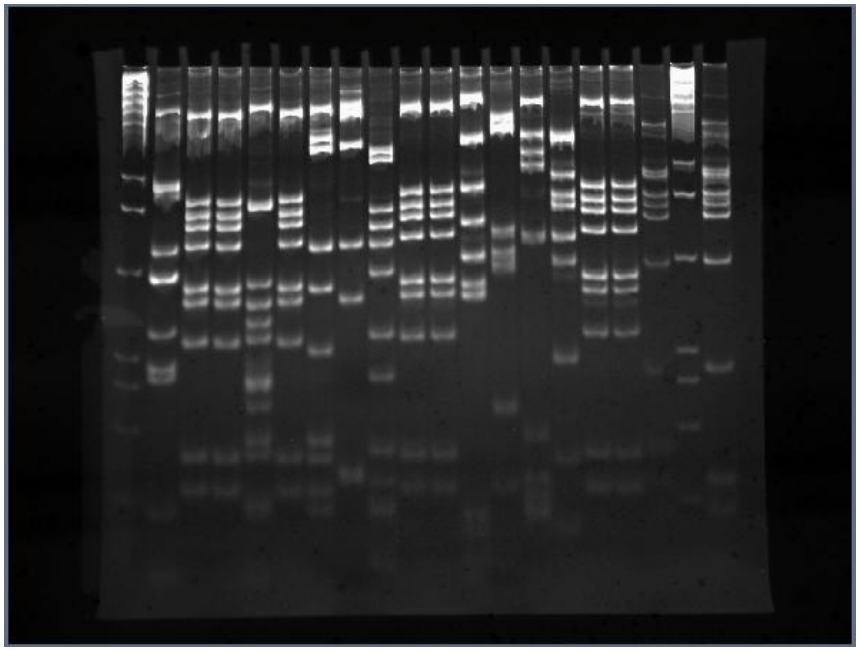
BLUE HOLE



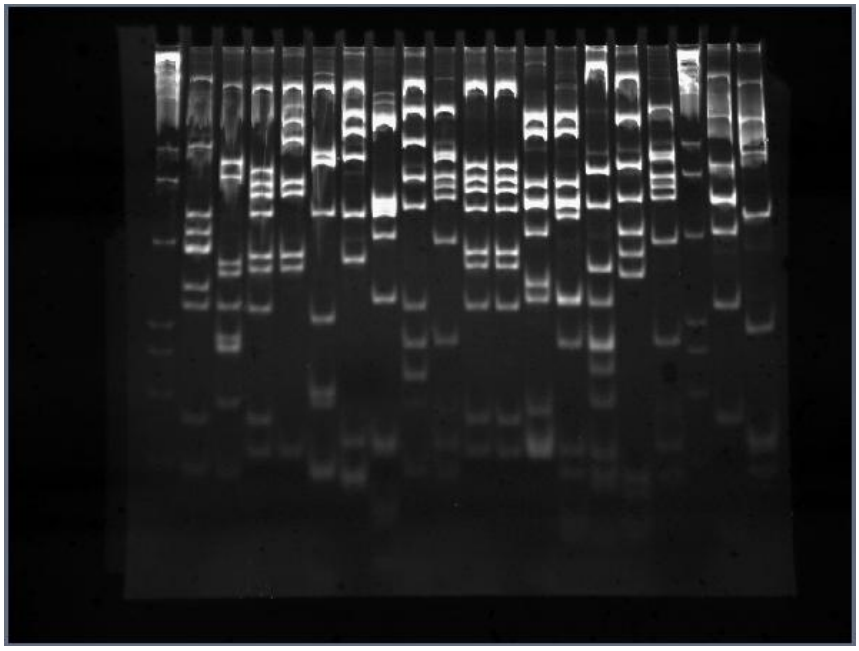
Gel 1 ARDRA patterns from clones 1-17; separated on an 8% acrylamide gel.



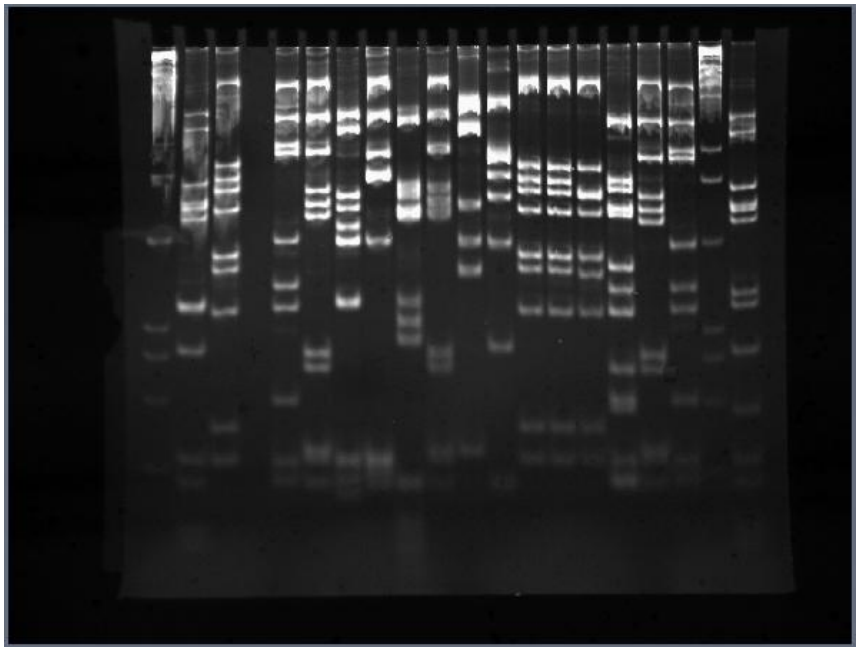
Gel 2 ARDRA patterns from clones 18-38; separated on an 8% acrylamide gel.



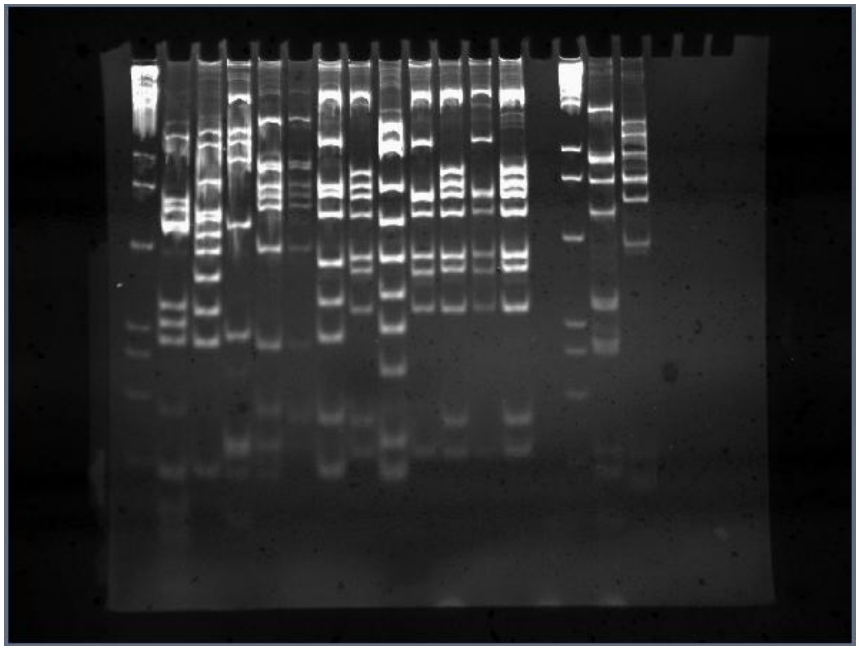
Gel 3 ARDRA patterns from clones 39-59; separated on an 8% acrylamide gel.



Gel 4 ARDRA patterns from clones 60-81; separated on an 8% acrylamide gel.



Gel 5 ARDRA patterns from clones 82-100; separated on an 8% acrylamide gel.



Gel 6 ARDRA patterns from clones 101-116; separated on an 8% acrylamide gel.

APPENDIX IV

MICROBIAL MAT DIVERSITY OF CLONED SAMPLES FROM CHEROKEE ROAD EXTENSION BLUE HOLE, ABACO ISLAND, BAHAMAS

Clone	Closest phyla	Accession number	Lowest defined lineage	Percent Similarity
CRE-MAT-1	Firmicutes	DQ811929	*Firmicutes bacterium*	93
CRE-MAT-3	Proteobacteria (delta/epsilon)	DQ303457	*Desulfoglaeba alkanexedens*	95
	Proteobacteria (delta/epsilon)	DQ303458	*Desulfoglaeba* sp. Lake	94
CRE-MAT-6	Uncultured	AB179679	uncultured bacterium	83
	Uncultured	EF019831	uncultured bacterium	91
CRE-MAT-7	Proteobacteria (delta/epsilon)	X70955	*Pelobacter acetylenicus*	87
	Proteobacteria (delta/epsilon)	DQ676442	delta proteobacterium	91
	Uncultured	DQ394922	uncultured bacterium	93
	Uncultured	AF154103	hydrocarbon seep bacterium	90
CRE-MAT-8	Gemmatimonadetes	DQ431883	*Gemmatimonadetes bacterium*	79
	Uncultured	AB177337	uncultured bacterium	82
	Uncultured	AB300068	uncultured bacterium	78
CRE-MAT-9	Proteobacteria (delta/epsilon)	AJ237607	*Desulfobacterium indolicum*	91
	Proteobacteria (delta/epsilon)	AF354164	delta proteobacterium	91
	Proteobacteria (delta/epsilon)	AJ535235	delta proteobacterium	92
	Proteobacteria (delta/epsilon)	EF061950	delta proteobacterium	90
CRE-MAT-15	Planctomycetes	DQ269110	uncultured planctomycete	84
	Uncultured	AF419673	uncultured bacterium	89
	Unidentified	AF317763	unidentified bacterium wb1_E15	84
CRE-MAT-17	Verrucomicrobia	AY114321	*Verrucomicrobia bacterium*	84
	Fibrobacteres	AB252949	*Fibrobacteres bacterium*	84
	Uncultured	DQ395743	uncultured organism	88

Clone	Closest phyla	Accession number	Lowest defined lineage	Percent Similarity
CRE-MAT-18	Proteobacteria (delta/epsilon)	AY155599	*Geothermobacter ehrlichii*	88
	Proteobacteria (delta/epsilon)	DQ811796	delta proteobacterium	89
CRE-MAT-20	Proteobacteria (delta/epsilon)	AY493562	*Desulfatibacillum alkenivorans*	92
	Proteobacteria (delta/epsilon)	AF148141	delta proteobacterium	92
	Uncultured	AY493563	sulfate−reducing bacterium	92
CRE-MAT-23	KSB1	DQ329547	candidate division KSB1	90
	OP11	AF424442	candidate division OP11	91
	Deferribacteres	DQ811937	*Deferribacteres bacterium*	94
	Uncultured	DQ394944	uncultured bacterium	89
CRE-MAT-24	Unidentified	AY344404	unidentified bacterium	93
CRE-MAT-26	Deferribacteres	DQ811937	*Deferribacteres bacterium*	94
	Uncultured	DQ394944	uncultured bacterium	89
CRE-MAT-27	Proteobacteria (delta/epsilon)	AY360496	uncultured proteobacterium	85
	OP3	AM712351	candidate division OP3	98
	OP3	AM712351	candidate division OP3	98
	Uncultured	EF602488	uncultured bacterium	86
	Uncultured	AF469396	uncultured bacterium	88
	Unidentified	AY344402	unidentified bacterium	87
CRE-MAT-30	OP11	DQ811940	candidate division OP11	84
	Uncultured	DQ154824	uncultured bacterium	90
	Uncultured	DQ404613	uncultured bacterium	87
	Uncultured	AB250572	uncultured bacterium	90
CRE-MAT-36	Deferribacteres	DQ811937	*Deferribacteres bacterium*	92
	Uncultured	DQ394944	uncultured bacterium	90
CRE-MAT-38	Proteobacteria (delta/epsilon)	AJ704688	delta proteobacterium	87
CRE-MAT-39	Proteobacteria (delta/epsilon)	DQ331004	uncultured proteobacterium	91
	Uncultured	DQ394922	uncultured bacterium	90

Clone	Closest phyla	Accession number	Lowest defined lineage	Percent Similarity
CRE-MAT-42	Proteobacteria (delta/epsilon)	DQ303457	*Desulfoglaeba alkanexedens*	91
	Proteobacteria (delta/epsilon)	DQ303458	*Desulfoglaeba* sp. Lake	91
CRE-MAT-44	Deferribacteres	DQ811937	*Deferribacteres bacterium*	92
	Uncultured	AM181829	uncultured bacterium	87
	Uncultured	DQ394944	uncultured bacterium	90
CRE-MAT-46	Chloroflexi	DQ330152	*Chloroflexi bacterium*	93
CRE-MAT-48	Unidentified	AY344404	unidentified bacterium	92
CRE-MAT-49	Proteobacteria (delta/epsilon)	AY177804	*Desulfuromonadales bacterium*	89
	Proteobacteria (delta/epsilon)	DQ676358	delta proteobacterium	91
CRE-MAT-51	TG2	AB192184	candidate division TG2	86
	Uncultured	AB100495	uncultured bacterium	87
	Uncultured	AB089068	uncultured bacterium	87
CRE-MAT-52	Proteobacteria (delta/epsilon)	AY542227	delta proteobacterium	94
	Uncultured	AF154102	uncultured bacterium	93
CRE-MAT-53	Proteobacteria (delta/epsilon)	DQ394958	*Geobacter* sp.,	92
	Proteobacteria (delta/epsilon)	AY711087	uncultured proteobacterium	95
CRE-MAT-54	Unidentified	AY344404	unidentified bacterium	93
CRE-MAT-56	Proteobacteria (delta/epsilon)	AJ704683	delta proteobacterium	92
CRE-MAT-57	Unidentified	AY344404	unidentified bacterium	92
CRE-MAT-59	TM6	DQ676372	candidate division TM6	88
	Uncultured	DQ394961	uncultured bacterium	88
CRE-MAT-60	Chloroflexi	DQ811873	*Chloroflexi bacterium*	94
CRE-MAT-61	Proteobacteria (delta/epsilon)	AY651787	*Syntrophobacter sulfatireducens*	91
	Proteobacteria (delta/epsilon)	DQ303457	*Desulfoglaeba alkanexedens*	91
	Proteobacteria (delta/epsilon)	DQ303458	*Desulfoglaeba* sp. Lake	91
	Proteobacteria (delta/epsilon)	AY965995	delta proteobacterium	96
	Proteobacteria (delta/epsilon)	AY965996	delta proteobacterium	91
CRE-MAT-64	Chlorobi	AM690798	*Prosthecochloris vibrioformis*	93
CRE-MAT-65	Verrucomicrobia	DQ676300	*Verrucomicrobia bacterium*	82
	Unidentified	AY792312	unidentified bacterium	83
	Unidentified	AY344402	unidentified bacterium	87

Clone	Closest phyla	Accession number	Lowest defined lineage	Percent Similarity
CRE-MAT-67	OP3	AM712351	candidate division OP3	85
	OP3	AM712351	candidate division OP3	85
	Uncultured	EF602488	uncultured bacterium	87
	Uncultured	AB252431	uncultured bacterium	88
CRE-MAT-68	KSB1	DQ329541	candidate division KSB1	87
	Deferribacteres	DQ811937	*Deferribacteres bacterium*	93
	Uncultured	DQ394944	uncultured bacterium	88
CRE-MAT-69	Uncultured	DQ154824	uncultured bacterium	93
	Uncultured	DQ404613	uncultured bacterium	89
CRE-MAT-70	Chlorobi	AJ428420	*Chlorobi bacterium*	92
	Chlorobi	AM690795	*Prosthecochloris vibrioformis*	92
CRE-MAT-74	Unidentified	AY344404	unidentified bacterium	93
CRE-MAT-75	Proteobacteria (delta/epsilon)	AJ535229	delta proteobacterium	94
CRE-MAT-76	Proteobacteria (delta/epsilon)	DQ112443	delta proteobacterium	89
	Uncultured	AY355303	uncultured bacterium	95
CRE-MAT-77	Proteobacteria (delta/epsilon)	CP000478	*Syntrophobacter fumaroxidans*	90
	Uncultured	DQ415855	uncultured bacterium	89
CRE-MAT-78	Proteobacteria (delta/epsilon)	AB188782	delta proteobacterium	89
	Uncultured	FJ716342	uncultured bacterium	90
CRE-MAT-80	Proteobacteria (delta/epsilon)	DQ676358	delta proteobacterium	91
CRE-MAT-81	Proteobacteria (delta/epsilon)	AJ704683	delta proteobacterium	92
CRE-MAT-82	Proteobacteria (delta/epsilon)	DQ811799	delta proteobacterium	92
	Uncultured	AF482433	uncultured bacterium	92
CRE-MAT-84	WS3	AY114311	candidate division WS3	83
	Unidentified	AY532589	unidentified bacterium	85
CRE-MAT-85	Proteobacteria (delta/epsilon)	AJ704683	delta proteobacterium	91
CRE-MAT-90	GN04	DQ330593	candidate division GN04	89

Clone	Closest phyla	Accession number	Lowest defined lineage	Percent Similarity
CRE-MAT-91	Proteobacteria (delta/epsilon) Uncultured	AJ704683 AY869687	delta proteobacterium uncultured bacterium	91 90
CRE-MAT-92	Planctomycetes Unidentified	DQ070819 AY532589	uncultured planctomycete unidentified bacterium	87 85
CRE-MAT-98	Proteobacteria (delta/epsilon)	AJ704683	delta proteobacterium	98
CRE-MAT-99	WS3 Fibrobacteres Unidentified	AY114311 AB252949 AY532589	candidate division WS3 *Fibrobacteres bacterium* unidentified bacterium	81 82 84
CRE-MAT-100	Uncultured	AY355303	uncultured bacterium	90
CRE-MAT-101	GN04	DQ330596	candidate division GN04	87
CRE-MAT-104	Verrucomicrobia Unidentified	DQ676384 CR933082	*Verrucomicrobia bacterium* unidentified bacterium	96 85
CRE-MAT-105	Proteobacteria (delta/epsilon)	AJ535248	delta proteobacterium	93
CRE-MAT-108	Verrucomicrobia Uncultured	DQ676384 AJ390463	*Verrucomicrobia bacterium* uncultured soil bacterium	89 92
CRE-MAT-110	Proteobacteria (delta/epsilon)	DQ811814	delta proteobacterium	96
CRE-MAT-115	Acidobacterium Uncultured	AJ241004 AY375053	Holophaga/Acidobacterium uncultured bacterium	81 81
CRE-MAT-116	Uncultured	AY568788	uncultured bacterium	96

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Education

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Research Experience

12/09	Field Technician TAMU-CONACYT	Piedras Negras, Mexico
09/08	Research Assistant *NSF*	Bayahibe, Dominican Republic
02/07	Exploration Team Member *ADM* Yucatán *Expedition*	Homun, Mexico
03/06	Field Assistant *NSF*	Abaco Island, Bahamas

Academic Professional Employment

08/05-07/10	Teaching Assistant, Marine Biology Teach: Scientific Diving, Tropical Marine Ecology, Biospeleology, Vertebrate Biology, and Biology	Galveston, TX
08/05-08/10	GISD/Sea Camp Instructor	Galveston, TX

Publications

Gonzalez BC, Iliffe TM, Macalady JL, Schaperdoth I, Kakuk B. (2010). Biogeochemistry and microbial diversity of anchialine blue holes from the Bahamas. Hydrobiologia (in press).